Bioanalysis of ibuprofen enantiomers : application to pharmacokinetic studies in young and elderly volunteers.

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ABSTRACT

(*R,S*)-Ibuprofen is an important non-steroidal anti-inflammatory drug widely used for the treatment of pain and inflammation associated with rheumatic disorders. The enantiomers of ibuprofen exhibit differential pharmacodynamic and pharmacokinetic properties *in-vivo* and *in-vitro*, and undergo metabolic chiral inversion from the inactive *R*- enantiomer to its active *S*- antipode. Although the enantiomeric disposition of ibuprofen in man has been extensively investigated, few studies have presented a comprehensive picture of the drug's disposition following the administration as a racemate, as a result of methodological difficulties in the determination of the unbound drug enantiomer concentrations and the determination of the stereochemical composition of its two major metabolites, hydroxyibuprofen and carboxyibuprofen.. Even less attention has been placed on the disposition of ibuprofen in one of the frequent group users, the elderly population. As such it is the objective of this investigation to examine the enantioselective disposition and pharmacokinetics of ibuprofen in young and elderly volunteers. To achieve this objective sensitive and robust analytical methods were developed and validated for the enantiospecific analysis of ibuprofen in serum and urine. These methods are based on the indirect approach to chiral chromatography, involving derivatisation with (*R*)-(naphthen-1-yl)ethylamide in the presence 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 1-hydroxybenzotriazole (HOBT) as coupling agents. The diastereomeric amides were then separated using a reversed phase C_{18} HPLC system. The sensitivity of the method enabled it to be adapted to measure unbound drug concentrations in serum following equilibrium dialysis and was validated by comparison with a published radiochemical method. The diastereoisomers of carboxyibuprofen were resolved on a Chiralpak AD chiral stationary phase and the assignment of the stereochemical configuration to the eluted peaks were achieved using a combination of metabolic studies, stereoselective synthesis and chromatographic separation. The order of elution of the diastereoisomers was found to be 2'*S*,2*R*- 2'*R*,2*R*-, 2'*R*,2*S*-, and 2'*S*,2*S*-. The chiroptical properties of the diastereoisomers were also determined and differences in CD bands were observed at the 225 nm region. The determination of the enantiomeric composition of

hydroxyibuprofen and carboxyibuprofen in urine was carried out using a sequential normal phase achiral-chiral phase method based on the Chiralpak AD CSP.

The serum kinetics of ibuprofen enantiomers showed significant differences in $t_{1/2}$, V_d and CL following the administration of the racemic drug (400 mg) to healthy young volunteers. Plasma protein binding was found to be stereoselective ($R > S$). Pharmacokinetic parameters based on unbound concentrations revealed that clearance by inversion was the main pathway of metabolism for the *R*-enantiomer, while clearance by oxidative pathways was stereoselective for the *S*-enantiomer. The metabolite formation clearances based on unbound drug enantiomer concentrations also reflected stereoselective formation of metabolites with the *S*- configuration in the propionic acid moiety. The stereoselectivity for the formation of the carboxyibuprofen diastereoisomers indicate that substrate-product stereoselectivity, i.e the 2'*R*,2*R*- stereoisomer was the preferred product for (*R*)-ibuprofen while the 2'*S*,2*S*- stereoisomer was the preferred product for (*S*)-ibuprofen. Ageing was found to have no significant effect on the pharmacokinetic parameters of the enantiomers of ibuprofen based on total serum concentrations. However, a significant increase in the free fraction was observed for (*S*)- but not (*R*)-ibuprofen in the elderly. In addition, ageing resulted in a significant decrease in the unbound clearance of (*S*)-ibuprofen but not in either the unbound inversion or non-inversion clearance of the *R*- enantiomer. The opposing effects of increased fraction unbound and reduced clearance of (*S*)-ibuprofen account for the lack of observed effect of ageing on ibuprofen disposition when pharmacokinetic parameters are based on total serum drug enantiomer concentrations. The implications of the increased free serum concentration of the active enantiomer are discussed.

Acknowledgements

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CHAPTER 1

Introduction

1. Introduction

1.1 Stereochemistry

Stereochemistry is the area of chemistry concerned with the three dimensional spatial arrangement of the atoms or groups within a molecule. The prefix stereo originates from the Greek *stereos* meaning solid. The origins of stereochemistry stem from the discovery of plane polarised light by Malus in 1809 and the observation that quartz, and later tartaric acid crystals rotate the plane of plane polarised light. It was also early in the 19th century that the ability to rotate plane polarised light i.e. optical activity was recognised as a property of crystals of compounds such as sodium chlorate and sodium bromate but not of the solutions. Much later in 1848, Pasteur extended this observation from crystals to the realm of molecules like tartaric acid which show optical activity in both the crystalline form and in solution. By crystallisation of sodium ammonium tartrate from aqueous solutions, he obtained two types of hemihydric crystals i.e crystals with facets arranged such that non-superimposible species were formed, similar to that of quartz crystals. After separating the crystals by hand, he re-dissolved them individually and found that one solution rotated polarised light to the left [(-)-tartrate] and the other to the right [(+)-tartrate]. This led him to the conclusion that optical activity was a characteristic of the molecule and that the individual molecules of (+)- and (-)-tartaric acid are stereochemically dissymmetric, related to each other as non-superimposible mirror images and expressed as hemihydric crystals in their sodium ammonium salt form. The two forms are known as enantiomorphs or enantiomers (Greek *enantios*, opposite; *morph*, form). Compounds which show this behaviour are said to be chiral (Greek *chiros*, handed), because like an individual's hands, the individual stereoisomers are not superimposible with their mirror images (Eliel and Wilen, 1994).

The most common source of asymmetry in molecules of interest in biology arises due to the presence of tetrahedral carbon atoms to which four different groups are bonded, such atoms are centres of asymmetry or chiral centres (Figure 1.1).

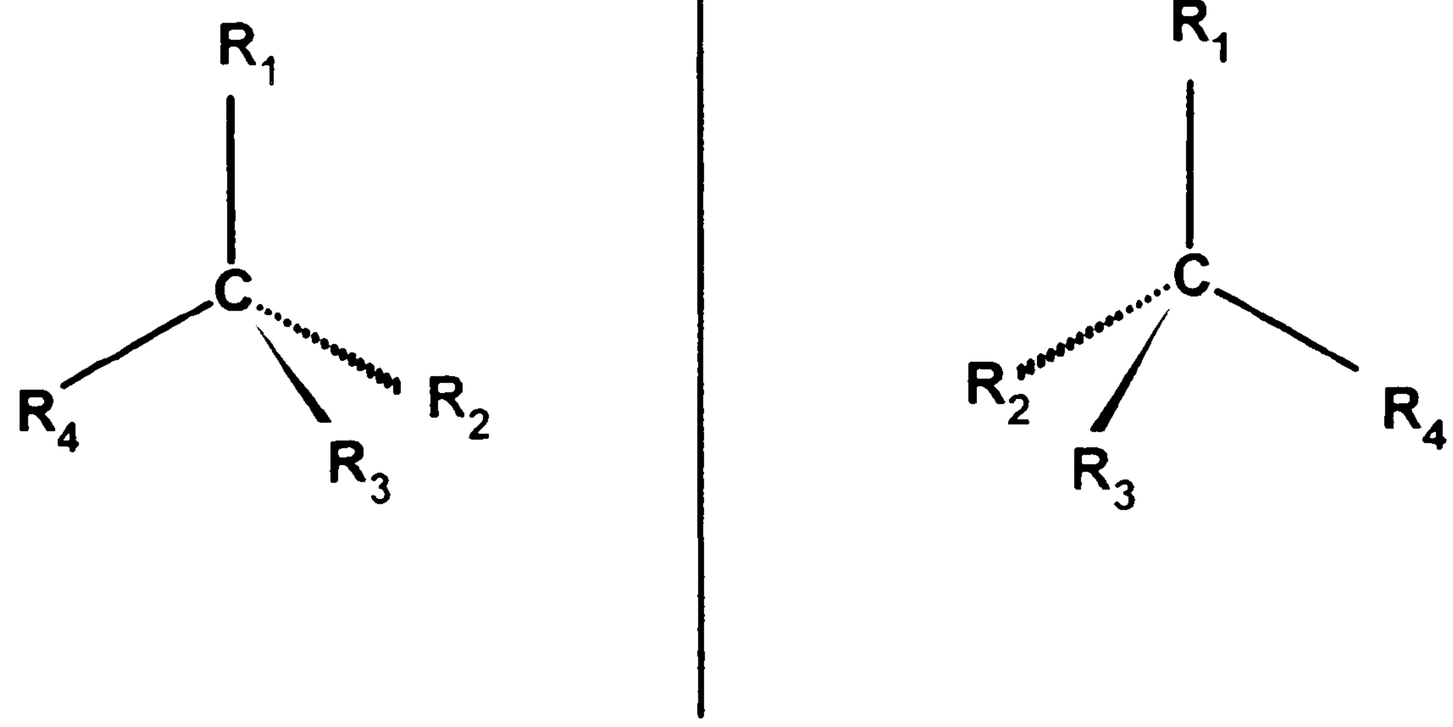
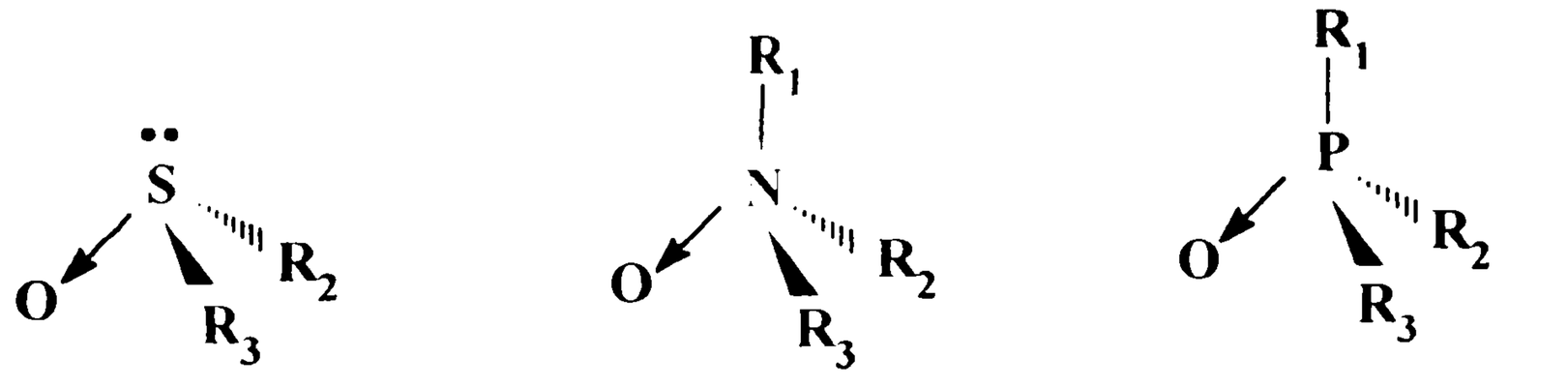


Figure 1.1: A pair of enantiomers based on an asymmetric carbon

Other atoms such as nitrogen, sulphur and phosphorus to which four different groups are bonded also yield chiral molecules of importance in pharmacology (Figure 1.2) (Allenmark, 1988).



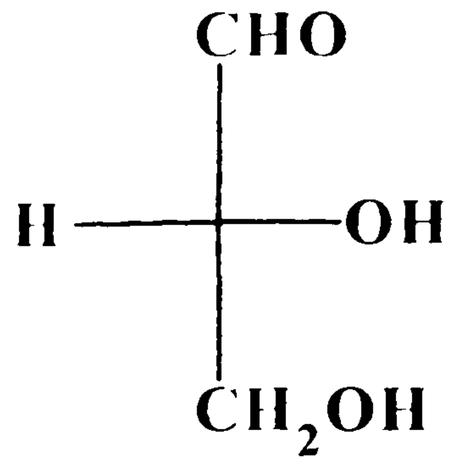
sulphoxide **tertiary amine N-oxide** **phosphine-oxide**

Figure 1.2: Examples of tetrahedral structures based on asymmetric atoms other than carbon.

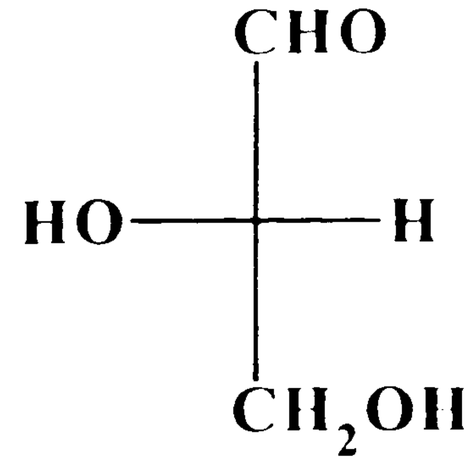
Although chiral compounds are usually based on an asymmetric centre, this is not essential as asymmetry can occur across an axis or a plane. however relatively few chiral compounds of this type are known to be of pharmacological importance.

1.2 Nomenclature

In the earliest studies of stereoisomerism, enantiomers were distinguished by their ability to rotate the plane of plane polarised light. Single enantiomers rotate the plane of plane polarised light either to the left or right of the centre line of a polarimeter. This phenomenon gave rise to the notations dextrorotatory (*d* or +) and laevorotatory (*l* or -) for the individual stereoisomers respectively. A racemic mixture, a 1:1 mixture of the two enantiomers, being indicated by either a *d,l* or (±) prefix to the name of the compound. This notation describes a physical property of the molecule and indicates whether a single isomer or mixture is present but does not describe the actual spatial arrangement of the atoms or groups around the chiral centre, i.e. it gives no information concerning the three dimensional spatial arrangement of the molecule. At the end of the 19th century, while structures could be represented in three dimensions it was not possible with the available technology to determine the actual structure of compounds and relate the observed optical rotations to a particular stereoisomeric structure. Three dimensional spatial arrangements, or configurations were arbitrarily assigned to the enantiomers of the carbohydrate glyceraldehyde such that the (+)-isomer was said to have the D-configuration and the (-)-isomer was said to have the L-configuration when drawn as Fischer projections (Figure 1.3).



D-(+)-glyceraldehyde



L-(-)-glyceraldehyde

Figure 1.3: Fischer projections of D-(+)-glyceraldehyde and L-(-)-glyceraldehyde

Using this system, the configurations of other compounds were related chemically to that of either of the reference compounds D-glyceraldehyde or L-serine, and were referred to as relative configurations. It was not until the 1950s that it was shown that (+)-glyceraldehyde had the structure assigned to it. Due to the difficulty of chemical transformation, the confusion between the small and capital letter notations (D and *d*; L or *l*) and the difficulties in applying the system in many situations, the notation should now be restricted for the designation of carbohydrates and amino acids.

The most commonly used system to designate stereoisomers is the Cahn-Ingold-Prelog system (Cahn *et al.*, 1956). Once the three dimensional structure of a stereoisomer has been determined by for example, X-ray crystallography, the substituent atoms around the chiral centre are assigned a priority according to their atomic number. If this fails to distinguish between the priority of two particular substituent groups, then the priority of the next atoms in the groups is considered, and so on until the ranking of the four groups has been assigned. The molecule is then viewed from the side opposite the lowest priority group. If the sequence of priority, highest to lowest, are arranged in a clockwise manner, then the chiral centre is designated the *R*- or rectus absolute configuration and if the sequence is

counterclockwise, the centre is designated the *S*- or sinister configuration (Figure 1.4).

***S*-enantiomer** ***R*-enantiomer**

A > B > C > D

Figure 1.4: Assignment of absolute stereochemical configuration according to the Cahn-Ingold-Prelog convention.

Additional rules concerning the designation of planar and axial symmetry are also included in this system (Cahn *et al.*, 1956).

1.3 Properties of enantiomers

While enantiomers have identical physical and chemical properties, they differ in two important aspects, the direction of rotation of plane polarised light (which is equal in magnitude) and different reaction rates with other chiral molecules. In chemical reactions with a second optically pure agent, the reaction transition states are diastereoisomeric (see below) and thus have different energies which gives rise to different reaction rates. Biological reactions are predominantly stereoselective, as enzymes, the all important catalysts of biological systems are chiral in nature. Thus (+)-glucose, but not (-)-glucose, is oxidised by animal and plant cells for energy. The fundamental principle is that enantiomers show different chemical and physical properties only in a chiral environment. Plane polarised light provides such an environment. The solubility, adsorption and reaction rates of

enantiomers may also be different in a chiral medium, but not in an achiral environment. Thus, enantiomers cannot be separated by ordinary chemical methods like fractional distillation, fractional crystallisation or chromatography, unless in the latter two methods, chiral solvents or chiral adsorbents are used.

When there is more than one chiral centre in a molecule, the number of stereoisomers possible are 2^n, where n is the number of chiral centres, with half that number of pairs of enantiomers. Those stereoisomers which are not enantiomeric are by definition diastereomeric pairs of compounds (Figure 1.5). Diastereomers have different physical properties and have similar but not identical chemical properties. Thus, in principle, diastereomers can be separated by fractional distillation, crystallisation and chromatography.

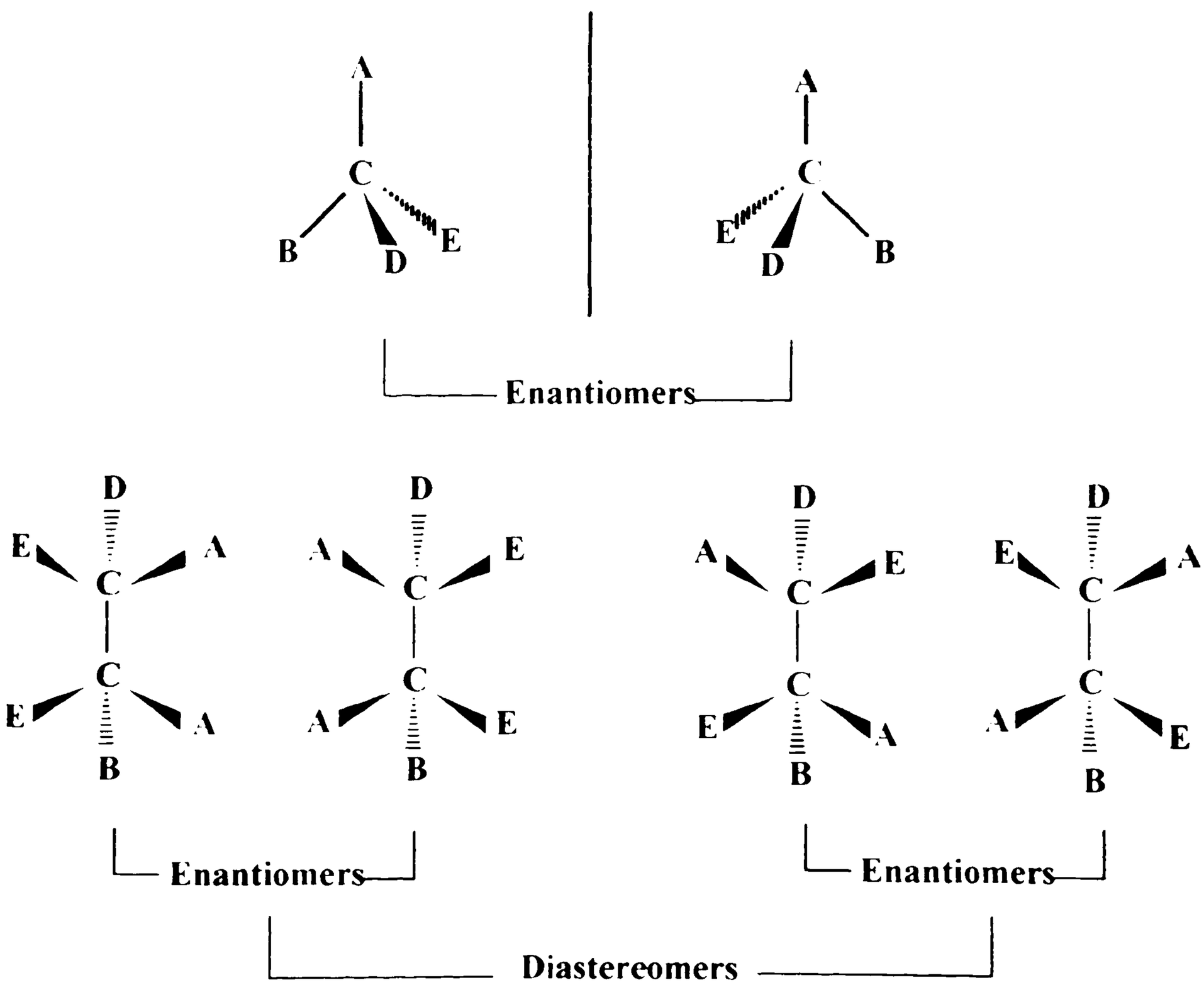


Figure 1.5: Relationships between enantiomers and diastereomers

1.4 Stereoselectivity in Pharmacology

Biological environments are invariably chiral as the structural, functional and catalytic components of biological systems are composed of chiral macromolecules, e.g. proteins, enzymes and receptors, from the chiral building blocks of L-amino acids and D-carbohydrates. Differential interactions between a chiral biological macromolecule and a pair of enantiomers are therefore expected as they are viewed as two different chemical entities. The differential pharmacodynamic activity of a pair of enantiomers has resulted in additional terminology such that the enantiomer with the greater affinity or activity is termed the eutomer and that with the lower affinity or activity is termed the distomer. The ratio of their activities, the eudismic ratio is a measure of the stereoselectivity of the system. Stereoselective differences in drug effects have long been known, e.g D-(-)-isoprenaline is 800 fold more potent as a bronchodilator than L- (+) isomer (Albert, 1985). The observed differences in biological activities between enantiomers arise as a result of their differential interactions at the receptor level. A biologically active molecule must complement the receptor at the site of action both sterically and chemically. Stereoselectivity in action arises when one enantiomer fits better at the receptor than its optical antipode. According to the universally accepted Easson-Stedman interaction model (Easson and Stedman, 1933), the more potent enantiomer can be involved with a minimum of three molecular interactions with the receptor site, while the less active enantiomer may take part in two interactions only (Figure 1.6). While the above model provides a means of understanding molecular interactions at the receptor level, it is a simplistic representation of a complex process. The model implies that drug molecules assume a particular orientation when interacting with the complementary receptor site. It is known that a change of conformation may occur in the drug molecule on contact with the receptor, and the converse is also true (Albert, 1985).

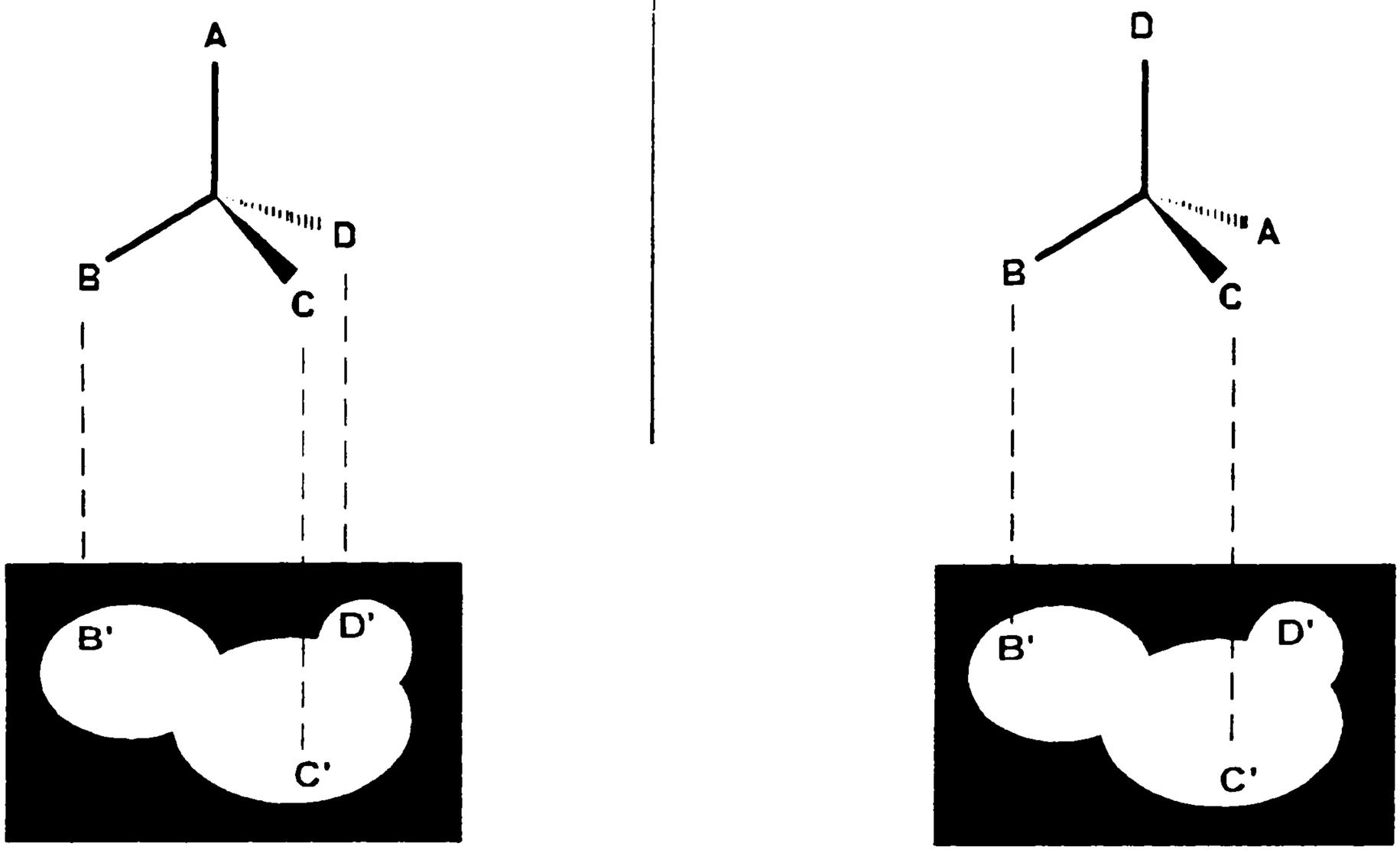


Figure 1.6: Interaction of two enantiomers with a receptor site.

The enantiomer on the left is the more potent, with three molecular interactions while that on the right interacts at two points only.

Recent advances in synthetic and analytical chemistry have facilitated investigations of the effects of individual enantiomers and this has led to a better understanding of the differences in their biological activity, and the factors that affect them. Evidence from such studies indicate that the pharmacological and toxicological properties of a racemic mixture are not necessarily the sum of the contributions of the two enantiomers. The situation is complex and it is important to understand the contribution made by each enantiomer to the observed activity. A variety of differential enantiomer contributions to pharmacological effects are known and these are outlined below.

a) Pharmacological activity resides only in one enantiomer.

Although it is a very desirable situation if the pharmacological activity resides only in one enantiomer, while the antipode is totally inactive, examples of this are not common. Usually the "inactive" isomer possess

some degree of activity. In the case of the antihypertensive α–methyldopa (Figure 1.7), the antihypertensive activity resides only in the *S*-enantiomer (Gillespie *et al.*, 1962) and the drug is marketed as a single isomer.

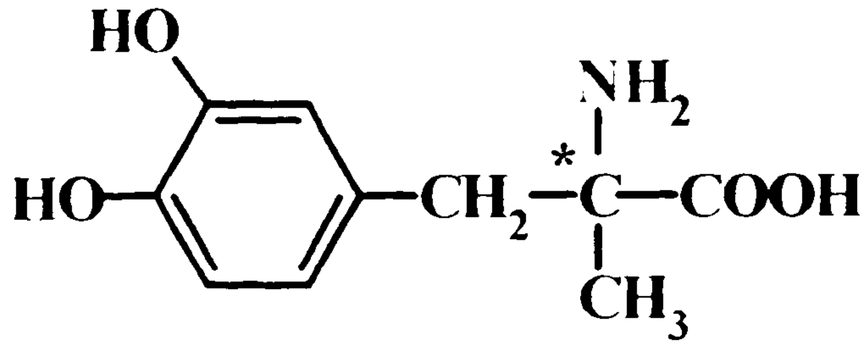
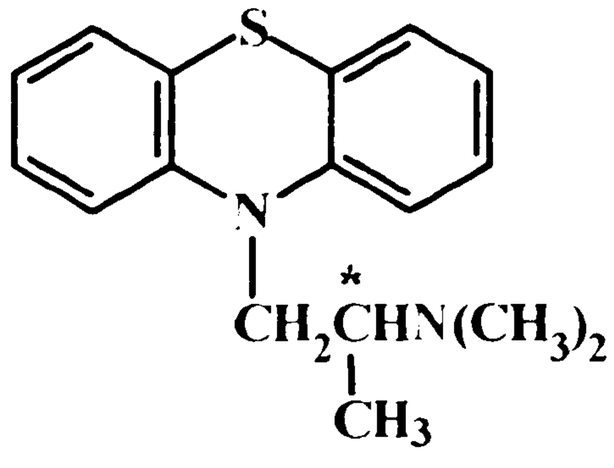


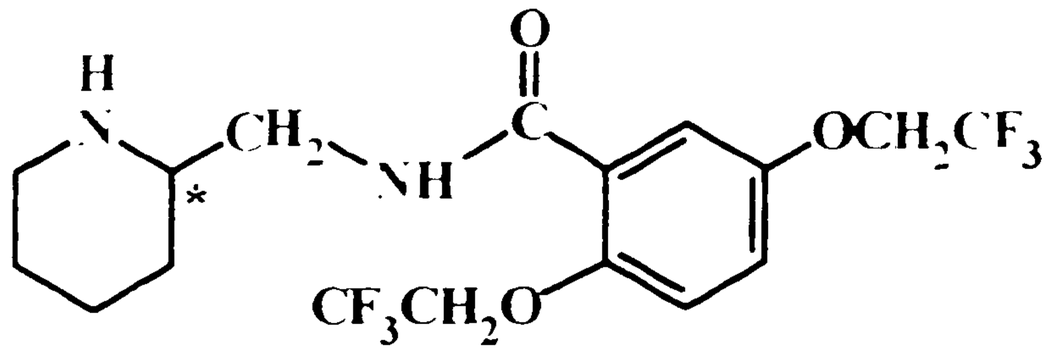
Figure 1.7: Structure of α-methyldopa

b) Both enantiomers are equally active.

Both enantiomers of promethazine (Figure 1.8) display similar pharmacological and toxicological properties (Powell *et al.*, 1988). The introduction of the chiral centre in the dimethylaminoethyl moiety results in a 100% increase in antihistaminic potency compared to the non-chiral analogue. The enantiomers of flecainide (Figure 1.8) are equipotent in their antiarrhythmic activity and effect on cardiac sodium channels and the pharmacokinetic properties of the individual enantiomers also appear to be similar (Kroemer *et al.*, 1989). Often enantiomers may display stereoselectivity with respect to one pharmacological effect but be equipotent in others. For example, the β-blocking effects of propranolol reside mainly in the *S*-enantiomer but its inhibition of thyroxine metabolism is not stereoselective (Heyma *et al.*, 1981).



(a)



(b)

Figure 1.8: Structures of a) promethazine and b) flecainide

c) Pharmacological activities of the enantiomers are qualitatively different

The classical example of this is situation is propoxyphene (Figure 1.9). Dextropropoxyphene has analgesic properties while the *l*-isomer has only antitussive effects (Drayer, 1986) and the enantiomers are marketed for different indications. To reflect their mirror image relationship they were given trade names which are also mirror images of each other i.e. Darvon and Novrad for *d*- and *l*-propoxyphene respectively. Similarly, dextromethorphan is a powerful antitussive and is virtually free from analgesic, sedative or other opiate-like effects while the antipode levomethorphan, has antitussive activity with potent opoid-like activity.

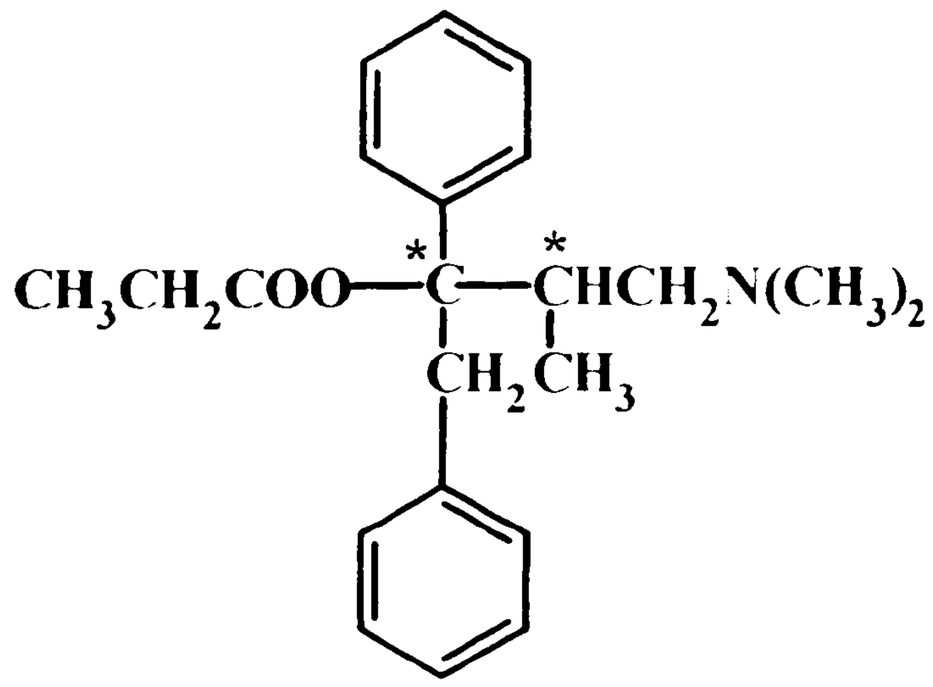
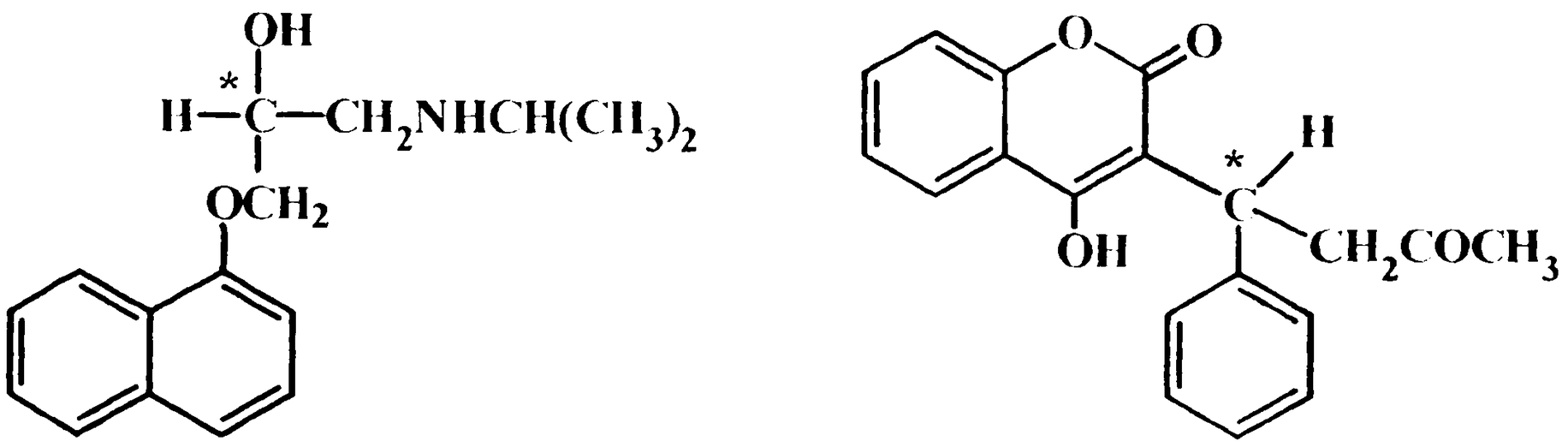


Figure 1.9: Structure of propoxyphene

d) Both enantiomers have similar activities but with different potencies.

This is the most common case for chiral drugs. The β-blocking activity of propranolol (Figure 1.10) and other aryloxypropanolamine derivative β–blockers resides principally in the *S*- enantiomers and the eudismic ratios vary from 100 for propranolol to 10 for atenolol (Walle *et al.*, 1988). However, with other pharmacological effects of propranolol e.g. the suppression of renin release and inhibition of thyroxine synthesis, no stereoselectivity was evident (Powell *et al.*, 1988). Other well known examples include warfarin (Figure 1.10), whose *S*- enantiomer is two to five fold more active in its anticoagulant effect than its *R*- antipode (O'Reilly *et al.*, 1974) and verapamil where the *S*- enantiomer is eight times more active in depressing cardiac activity (Echizen *et al.*, 1985).



(a) **(b)**

Figure 1.10: Structure of a) propranolol and b) warfarin

e) Both enantiomers are active but toxicity resides in one enantiomer.

Ketamine (Figure 1.11) is a useful anaesthetic with analgesic properties and does not cause circulatory or respiratory depression. However, the drug is addictive and adverse reactions such as post-anaesthesia reactions i.e hallucinations and agitation are common. (*S*)-Ketamine is 3.4 times more potent as an anaesthetic than the *R*-antipode but incidence of adverse effects i.e. psychic emergence reactions and post-operative agitated behaviour are greater with the *R*- enantiomer (White *et al.*, 1980). For dopa, many of the serious side effects like granulocytopenia are associated with the D-isomer

and as such the drug is marketed as the single L-isomer (Cotzias *et al.*, 1969).

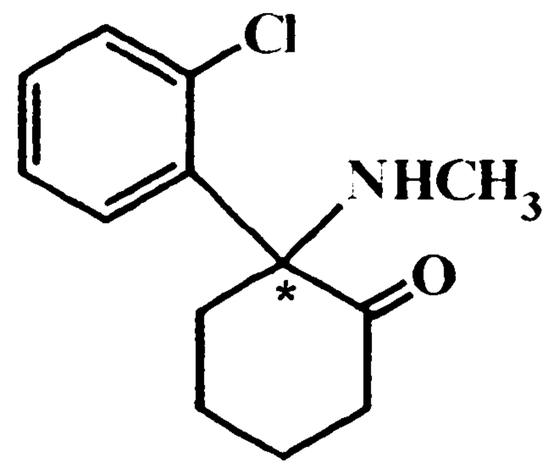


Figure 1.11: Structure of ketamine

f) The individual enantiomers evoke opposite or contrary effects.

The best example of this situation is indacrinone (Figure 1.12), a loop diuretic which shows both diuretic and uricosuric activity. Most diuretics increase plasma uric acid levels, which represents an additional risk factor in hypertensive states. When administered as a racemate, indacrinone also causes uric acid levels to increase. However, evaluation of the pharmacological activities of the individual enantiomers reveal that (*R*)- indacrinone is responsible for the diuretic activity and the *S*- antipode is responsible for uric acid secretion (Vlasses *et al.*, 1981). In man the plasma half-life of the *S*-enantiomer is much shorter than that of its *R*-antipode (half-life of 2 to 5 hr; compared to 10 - 12 hr for the *R*- enantiomer) and hence following the administration of the racemate, the uricosuric activity is too short to prevent the increase in serum uric acid. Thus, indacrinone may be a useful diuretic if the proportion of the *S*- enantiomer is increased (Tobert *et al.*, 1981).

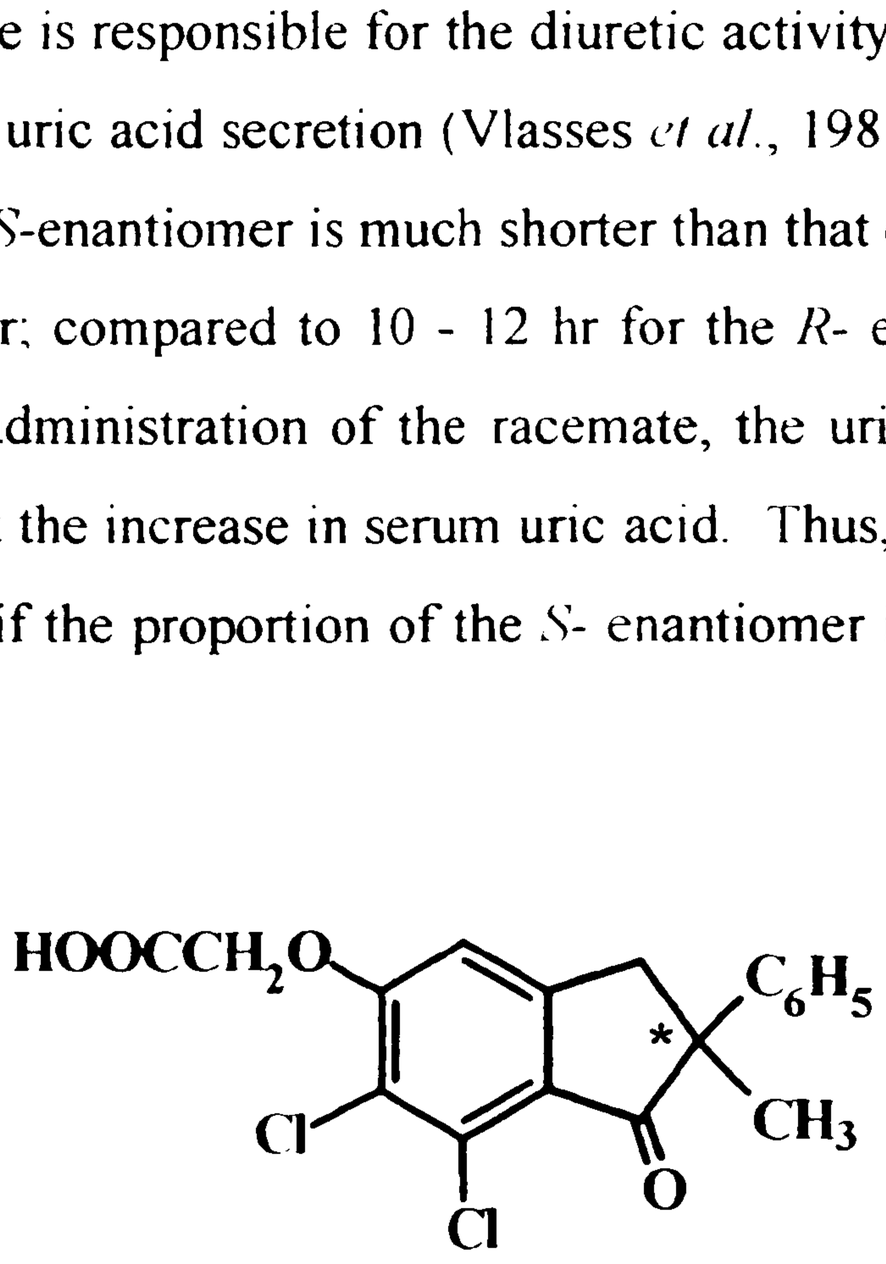


Figure 1.12: Structure of indacrinone

g) One enantiomer acts as antagonist to the other at the same receptor site

Picenadol (Figure 1.13) is a phenylpiperidine derivative that has both opioid agonist and antagonist activity. The analgesic properties principally reside in the (+) - enantiomer but the (-) - enantiomer is an antagonist, showing 1/10 the potency of naloxone as an opioid antagonist (Powell *et al.*, 1988). The racemate acts as a partial agonist due to the more potent activity of the (+)-enantiomer at the μ opioid receptor as compared to the weaker antagonist activity of the antipode at the same receptor.

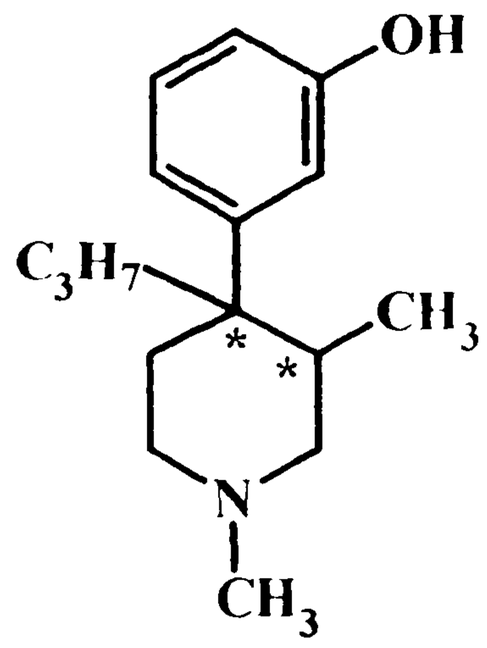


Figure 1.13: Structure of picenadol

It is thus clear that racemic mixtures are more than just mixtures with half the dose of the active drug, or containing inert ballast. To some the use of racemates is regarded as "polypharmacy" and inclusion of the "inactive" enantiomer regarded as having "50% impurity" in the dosage form (Ariens, 1984; Ariens, 1986). To date there is a large body of information regarding the stereoselective differences in pharmacodynamic activity of drug enantiomers which supports the view that the use of racemic mixtures may have important pharmacological and toxicological implications. This data has prompted a re-evaluation of drugs currently used as racemates and a number are being investigated for re-introduction as single isomer products.

1.5 Stereoselectivity in pharmacokinetics and drug disposition

Stereoselectivity in drug action is usually attributed to qualitative and quantitative differences in pharmacodynamic properties. However, many observed differences may be a result of stereoselectivity in pharmacokinetics (Tucker and Lennard, 1990; Levy and Boddy, 1991). Stereoselectivity may occur in virtually all phases of drug disposition i.e. absorption, distribution, metabolism and excretion. However, compared with the large magnitude of differences between enantiomers in their pharmacodynamic activity, the differences in pharmacokinetics tend to be relatively smaller, frequently 1 to 3 fold (Tucker and Lennard, 1990). However, these differences are of clinical significance.

a) Absorption

Generally drugs are absorbed from the gastro-intestinal tract (GIT), the airway passages or through the skin by passive diffusion, a process which depends upon the physico-chemical properties of the material, e.g. pKa, lipid solubility, molecular size. Since enantiomers do not exhibit differences in their physical properties, absorption is usually not stereoselective. However, where absorption occurs via active transport mechanisms, stereoselectivity has been observed. Thus the absorption of L-dopa and L-methotrexate is favoured over their respective D- antipodes (Wade *et al.*, 1973; Hendel and Brodhagen, 1984; Williams and Lee, 1985) as the L- isomers are absorbed by active transport mechanisms while the D- antipodes are absorbed by passive diffusion. With cephelexin, the L-isomer is preferentially absorbed by the intestinal dipeptide transport system, but only the D-isomer is detected in plasma as the L-isomer is also more susceptible to hydrolysis by peptidases in the gut wall (Tamai *et al.*, 1988). With drugs like terbutaline, other mechanisms may be the cause of the stereoselective difference in absorption. It has been suggested that (-)-terbutaline selectively promotes intestine membrane permeability for its (+)-antipode, thereby increasing its absorption (Borgstrom *et al.*, 1989). With local anaesthetics like bupivacaine, the enantiomers

display differential effects on local blood flow, which may account for the different rates of absorption (Alps and Reynolds, 1978).

b) Distribution

Most drugs penetrate biological membranes via passive diffusion and as such are not stereoselective in nature. As with absorption, where the penetration process is carrier mediated and energy dependent, stereoselectivity is evident. Stereoselective distribution as a result of stereoselectivity in tissue binding of the (*S*)-propranolol has been reported in dogs (Bai *et al.*, 1983). Stereoselective uptake, storage and secretion of (*S*)-propranolol and (*S*)-atenolol by adrenergic nerve terminals in cardiac tissue has also been reported (Walle *et al.*, 1988). The distribution of ibuprofen in perinephric fat (Williams *et al.*, 1986) and fenoprofen in rat hepatocytes (Sallustio *et al.*, 1988) favours the *R*-enantiomer. However, this selectivity in distribution is a result of the stereospecific formation of the acyl-CoA thioesters of the *R*-enantiomers of these agents followed by incorporation as hybrid triglycerides (see section 1.8.4).

It is generally accepted that it is the free or unbound drug that is responsible for pharmacological activity and available for clearance. Thus, the plasma protein binding of drugs is an important factor influencing pharmacodynamics and pharmacokinetics. The majority of drugs bind reversibly to plasma proteins, notably albumin and/or α_1-acid glycoprotein (AGP). For highly protein bound drugs, minor differences in binding may have significant effects on unbound drug concentrations and hence pharmacological action. Similarly, small differences in the plasma protein binding characteristics of enantiomers may result in significant differences in their pharmacodynamic and pharmacokinetic properties. Albumin preferentially binds acidic drugs and two ,binding sites are known. Of the two, site II (the benzodiazepine site) usually shows greater stereoselectivity than site I (the warfarin site) (Fehske *et al.*, 1981). α_1-Acid glycoprotein preferentially binds basic drugs and only one binding site is recognised. Stereoselective binding for verapamil (+ > -), diisopyramide (+ > -), mexilitine (- > +) and methadone (+ > -) have been reported

(Jamali *et al.*, 1989). Stereoselectivity in protein binding for one protein may differ from another, e.g. the protein binding of propranolol towards α_1-AGP is $S > R$, while for human serum albumin is $R > S$ (Hutt *et al.*, 1989). In whole plasma, the binding to α_1-AGP is of greater significance such that the *R*-enantiomer content of the free drug fraction exceeds that of (*S*)-propranolol.

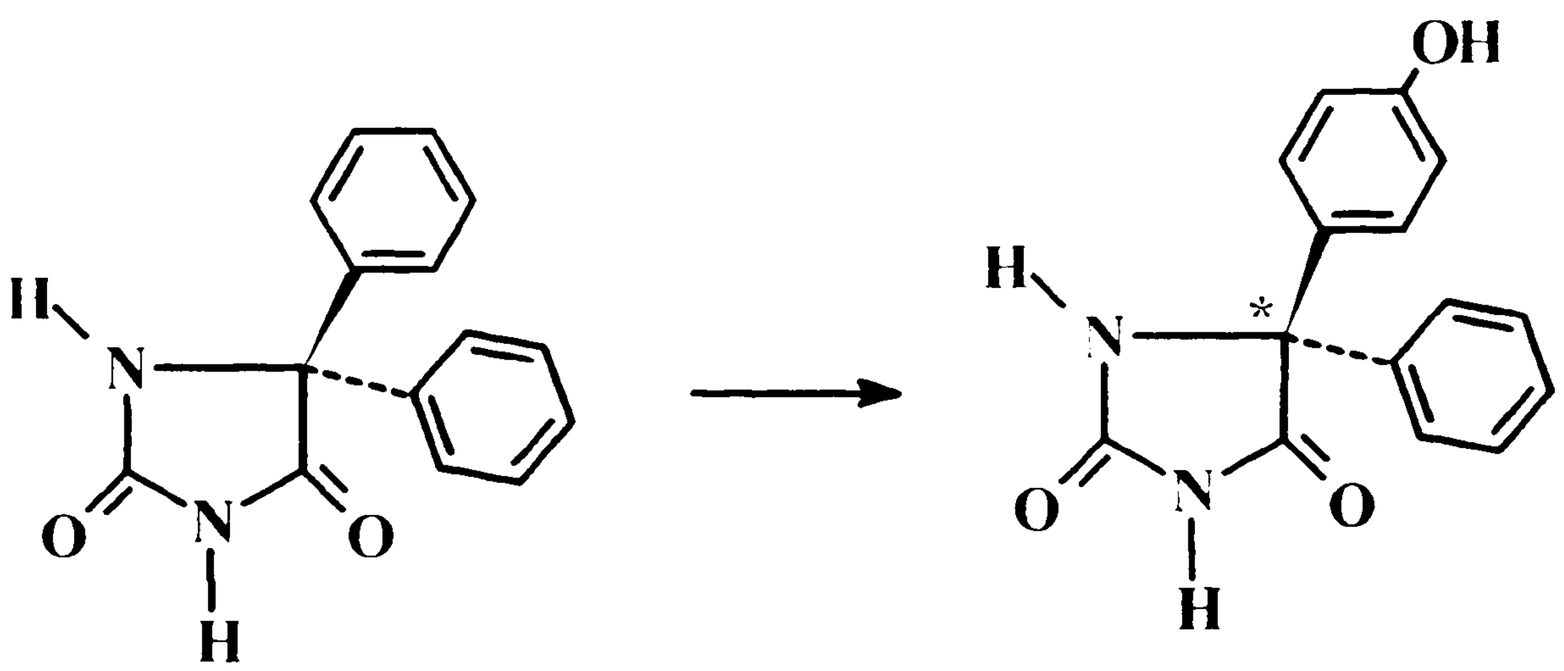
Competition for plasma protein binding sites is a common cause of enantioselective drug interactions. Sulphinpyrazone enhances the antiprothrombin effect of racemic warfarin by inhibiting the oxidation of (*S*)-warfarin, thereby decreasing total body clearance. However, sulphinpyrazone also stereoselectively displaces (*R*)-warfarin from plasma protein binding sites, causing an increase in clearance. The net effect is that the pharmacokinetics of racemic warfarin are unchanged but the pharmacological effect is increased (Toon *et al.*, 1986). In the case of disopyramide, the enantiomers show no differences in their kinetic properties following their individual administration. However, when administered as a racemate, the *S*-enantiomer shows reduced clearance, longer half-life ($t_{1/2}$) and smaller volume of distribution (V_d) compared to the *R*-enantiomer. This difference arises as a result of enantiomer-enantiomer interactions in the concentration dependent plasma protein binding of the drug (Giacomini *et al.*, 1986).

c) Drug metabolism

Stereoselectivity is commonly observed with both phase I and II metabolic reactions (Caldwell *et al.*, 1988a). Enantiomers may be metabolised by different routes to yield different products, or at different rates to give different isomeric compositions of the product. Enantioselective metabolism is best considered from the point of view of the stereochemical transformations involved:

i) Prochiral to chiral transformations

A prochiral molecule contains at least one site where a metabolic change occurs to yield a product that is chiral. Product stereoselectivity is observed where one enantiomeric form of the metabolite is favoured over the other. One of the most frequently cited examples is the biotransformation of diphenylhydantoin (phenytoin) to its para-hydroxylated metabolite (Figure 1.14). In man 90% of the metabolite is excreted as the *S*-enantiomer (Poupaert *et al.*, 1975), whereas in dogs, the *R*-enantiomer predominates. However the major metabolic pathway in dogs is meta-hydroxylation the *R*-form being predominant (Maguire, *et al.*, 1978). Thus, the transformation shows species dependent stereoselectivity as well as regioselectivity.

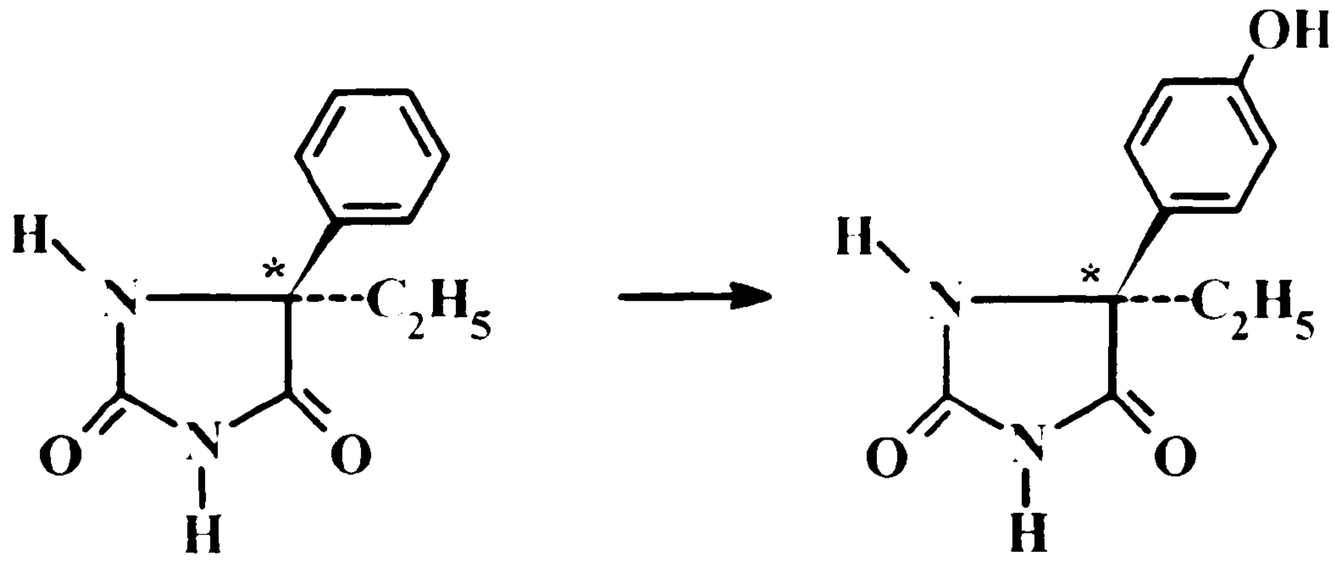


Diphenylhydantoin **(*S*)-4-hydroxydiphenylhydantoin**

Figure 1.14: Prochiral to chiral biotransformation of diphenylhydantoin.

ii) Chiral to chiral transformations

In this case the original chiral centre is retained in the molecule, metabolism taking place at a site remote from the chiral centre. With (*S*)-nirvanol, para-hydroxylation again occurs in man to yield the corresponding *S*- para-hydroxylated metabolite (Figure 1.15). The reaction is substrate selective for the *S*-enantiomer of the drug.



(*S*)-nirvanol **(*S*)-4-hydroxynirvanol**

Figure 1.15: Chiral - chiral biotransformation of nirvanol.

iii) Chiral to achiral transformations

Here, the chiral centre is lost on metabolism. Although not frequently encountered, it is nevertheless important especially if substrate stereoselectivity is observed. With rabbit liver microsomes, amphetamine undergoes hydroxylation at the α-carbon, followed by deamination to form phenylacetone (Figure 1.16) The reaction appears to be substrate stereoselective, the *R*-enantiomer being metabolised at a faster rate than the *S*- antipode (Wright *et al.*, 1977).

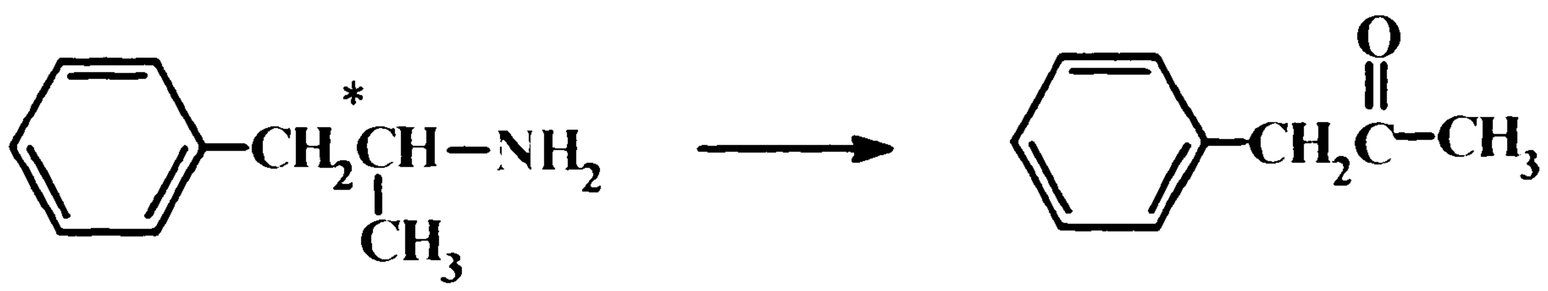


Figure 1.16: Oxidative deamination of amphetamine to phenylacetone.

iv) Chiral to diastereomer transformations

In this transformation, a new chiral centre is introduced into the molecule, thus a mixture of metabolites of varying diastereomeric composition are

possible. One of the best known examples is pentobarbitone. The 3'-hydroxylation of pentobarbitone in dogs is not substrate selective, and therefore similar amounts of the 3'-hydroxy metabolites are formed from both enantiomers. However, a second chiral centre is generated at the 3' position. The oxidation of the *R*-enantiomer yields equal quantities of 1'*R*,3'*S*- and 1'*R*,3'*R*- diastereomers while the *S*- enantiomer yields the 1'*S*,3'*R*- and 1'*S*, 3'*S*- diastereomers in a 5:1 ratio (Figure 1.16) (Palmer *et al.*, 1970). Metabolic conjugation with some amino acids (e.g. glutamine), glutathione and glucuronic acid involves reaction of a chiral substrate with a chiral endogenous molecule and thus diastereomers are formed in the process.

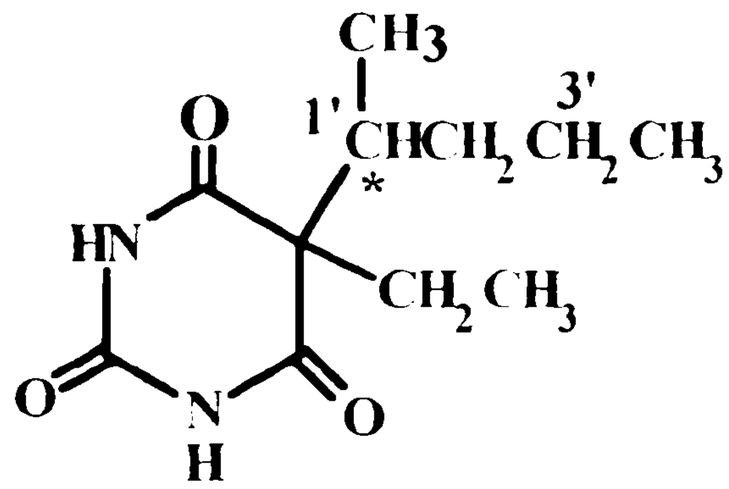


Figure 1.17: Structure of pentobarbitone

v) Chiral inversion

The inversion of a chiral centre involves the breaking of one of the bonds between the chiral centre and one of the groups attached to it, and migration of the leaving group to the other side of the molecule followed by subsequent reformation of the bond. Due to the chemical complexity of the situation involved, it is not surprising these transformations are of restricted occurance in drug metabolism. The inactive (*R*)-enantiomers of the 2-arylpropionic acids (2-APAs) undergo chiral inversion *in-vivo* to their *S*- antipodes in many animal species, including man (Hutt and Caldwell, 1983). The mechanism is thought to proceed via stereoselective formation of (*R*)-profenyl-CoA-thioesters that subsequently epimerize and hydrolyse to give either the *R*- or *S*- enantiomer. The *S*-enantiomer does not form the corresponding thioester,

and thus the inversion is unidirectional (Nakamura *et al.*, 1981). The chiral inversion of the *R*-enantiomers of the 2-APAs will be discussed in greater detail in section 1.8.4.

d) Renal excretion

Renal excretion involves three mechanisms, i.e. passive glomerular filtration, active secretion, active and passive reabsorption. Observed stereoselectivity in renal excretion may be a result of stereoselective plasma protein binding, resulting in differences in glomerular filtration and passive reabsorption. To demonstrate intrinsic stereoselectivity in renal excretion requires the calculation of the renal clearance of the unbound drug enantiomers. Stereoselectivity is usually associated with active secretion and this is thought to be responsible for the stereoselective clearance of pindolol (*S* > *R*), chloroquine (+ > -) and disopyramide (*S* > *R*) (Tucker and Lennard, 1990). It is most likely that stereoselective differences arise from the process of active secretion, although active reabsorption and renal metabolism may occur. Stereoselective renal tubular reabsorption is believed to be responsible for the stereoselective clearance of the *S*- enantiomer of terbutaline (Borgstorm *et al.*, 1989). Generally, the stereoselective differences in renal elimination are relatively small compared to those observed with non-renal clearance processes.

From the above, it is therefore apparent that stereoselectivity in pharmacokinetics is most significant for those processes that depend on the interaction between the stereoisomers with a chiral biological macromolecule e.g. binding to plasma proteins, transport by active transport carrier molecules and drug metabolism. It has been observed that the stereoselectivity observed for a particular pharmacokinetic parameter depends on the level of body organisation it reflects i.e. whether it is at macromolecular, organ or whole body level. Generally, the degree of stereoselectivity is maximum with those parameters concerned with processes at macromolecular level e.g protein binding and intrinsic formation clearances.

Stereoselectivity is at an intermediate level for whole organ parameters e.g. hepatic clearance and renal clearance as these parameters represent the net effects of several primary interactions between stereoisomers and macromolecules. For parameters involved at the whole body level, stereoselectivity is at a minimum e.g. half-life and total body clearance as these parameters reflect processes associated with multiple organs and are determined by the specific physiological relationships between the organs (Levy and Boddy, 1991). As a result of the stereoselectivity in the various processes of drug disposition, the plasma profiles of a drug administered as a racemate may differ markedly from when enantiomers are administered separately. Thus an estimation of pharmacokinetic parameters and concentration-effect relationships based on total drug concentrations are of limited value and potentially misleading, and has been described as "sophisticated nonsense" (Ariens, 1984).

1.6 Other factors affecting the stereoselectivity in both drug action and disposition.

It is apparent that significant differences exist in the pharmacokinetic and pharmacodynamic properties of the drug enantiomers and the possibilities of drug interactions are increased with chiral compounds especially if administered as racemates. Other factors that are known to affect the pharmacological properties of enantiomers include age, sex, disease, genetic predisposition and route of administration.

a) Disease

Drugs which undergo hepatic metabolism are frequently affected by liver dysfunction. Liver cirrhosis has been reported to alter the enantiomeric disposition of ibuprofen, and the elimination half-lives of both enantiomers were increased two-fold as compared to healthy volunteers (Li *et al.*, 1993). In the cirrhotic group of patients, the plasma concentrations of the active *S*- enantiomer were less than those of the *R*- antipode, a situation which is the reverse of that found in healthy

volunteers. The pharmacokinetics of ibuprofen are complicated by metabolic chiral inversion of the *R*- to the *S*- enantiomer but from the data presented in the report by Li *et al.*, (1993), it is not clear if the inversion process is impaired or that the relative increase in the (*R*)-ibuprofen concentrations is a result of stereoselective impairment of oxidative metabolic pathways, or both (Li *et al.*, 1993). Renal dysfunction has also been known to affect ibuprofen disposition by increasing the plasma *S R* ratios, which may further aggravate renal toxicity (Chen and Chen, 1995).

Oral administration of verapamil to patients with liver cirrhosis is associated with therapeutic effects being observed at lower plasma concentrations of racemic drug than with normal volunteers (Somogyi *et al.*, 1981). This is attributed to a stereoselective reduction in first-pass metabolism (Eichelbaum, 1988).

b) Age and Gender

Although age associated differences in drug disposition are well established age related differences in the enantiomeric disposition of chiral drugs is a relatively new phenomenon brought about by the recent awareness of the significance of drug chirality. The plasma clearance of (-)-hexobarbitone after oral administration is about two fold greater in the young as compared to the elderly, whereas that of the (+)-enantiomer showed no age associated difference (Chandler *et al.*, 1988). Sex and age related differences have been observed with methylphenobarbitone. In both young and elderly volunteers of both sexes, the *R*-enantiomer is more rapidly cleared as compared to the *S*- antipode. However, the clearance of the *R*-enantiomer in young males is significantly greater than that found in either young females or elderly males. Also, no significant differences are observed between young and elderly females or elderly males and females, indicating that the clearance of the *R*-enantiomer is both age and gender dependent. With the *S*-enantiomer, a shorter elimination half-life was observed for young males only, and this difference appears to arise as a result of differences in distribution, as no differences in clearance were observed with either age or sex. (Hooper and Qing, 1990).

c) Genetics

When a chiral drug which is a substrate for enzyme(s) that are known to exhibit genetic polymorphism is administered as a racemate, the enantiomeric composition in plasma as well as the plasma concentrations may vary with phenotype, thus influencing the clinical response to the agent. The β-blocker metoprolol is a known substrate for the CYP 2D6 isoenzyme and examination of the "total" drug plasma level-effect relationship following the administration of the racemate indicates that the drug is less effective in the poor metabolisers (PMs) compared to extensive metabolisers (EMs) phenotype. However, examination of the individual enantiomer profiles indicates that the plasma concentrations and the area under the plasma concentration-time curve (AUC) of active (*S*)-metoprolol is greater than its antipode in EMs, but the reverse is true in PMs. Thus, the shift of the concentration-effect curve to the left for EMs is because of a greater proportion of the total plasma concentration is due to the active *S*- enantiomer (Lennard *et al.*, 1983).

d) Route of administration

The "total" drug concentration-effect relationship (PR- interval prolongation) of verapamil indicates that the drug is more potent when administered intravenously as compared to oral administration as the oral "total" concentration-effect curve is shifted to the right. The explanation for this shift is the result of stereoselective first-pass metabolism. The first-pass metabolism is stereoselective for the more active *S*- enantiomer as compared to the *R*-antipode, leading to an enrichment of the less active enantiomer after oral administration of the racemate. Thus, for the same "total" plasma concentration, the proportion of the more active *S*- enantiomer is approximately 2.5 fold less following oral administration as compared to intravenous dosing (Eichelbaum, 1988). With propranolol, a similar mechanism occurs but with opposite effects. In this case, the first-pass metabolism is stereoselective for the less active *R*-enantiomer, leading to an opposite shift of the concentration effect curve, indicating that the drug is more potent following oral

administration as compared to intravenous administration. This effect is also enhanced by the formation of the active metabolite 4-hydroxypropranolol as a result of the first-pass metabolism (Walle *et al.*, 1988).

1.7 The importance of studying enantioselective pharmacokinetics

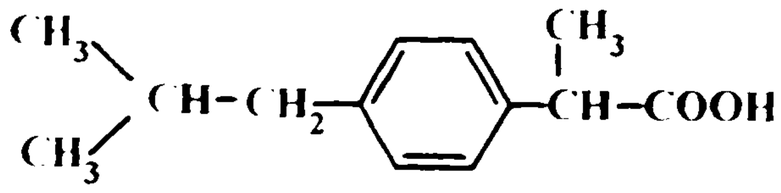
It is obvious from the discussion above that stereoselective differences in the pharmacodynamic and pharmacokinetic properties of stereoisomers seem to be the rule rather than the exception, and that administration of chiral drugs as racemates has far reaching pharmacological and toxicological implications. The strength of evidence has prompted regulatory agencies to promote the use of single isomer preparations for new chemical entities unless it can be proven that the racemate gives therapeutic benefits over the single enantiomer or that the preparation of a single isomer is not technically feasible on an industrial scale. An additional consideration would also be the risk-benefit ratio of a novel compound. For example, if a new agent were to be introduced for the treatment of AIDS or cancer, then the significance of a racemic mixture or single enantiomer may become somewhat "academic". However, for the majority of compounds still in use, racemates continue to be marketed and used. As such the continuing study of the pharmacokinetic and pharmacodynamic properties of such drugs, and their therapeutic re-evaluation is of immense importance in rational therapeutics as it is clear that previous non-stereoselective studies are of limited value and do not reveal underlying problems (Ariens, 1990). This is especially so where stereoselectivity is observed at more than one aspect of drug disposition. The 2-arylpropionic acids are a prime example of such types of drug, displaying stereoselectivity in protein binding, chiral inversion and enantioselective oxidation and conjugation. Moreover, they are disproportionately used in the elderly population (Johnson *et al.*, 1993), where changes in drug disposition have been widely reported, and where multiple drug administration and co-morbid disease states are common .

1.8 The 2-arylpropionic acid (2-APA) group of anti-inflammatory drugs

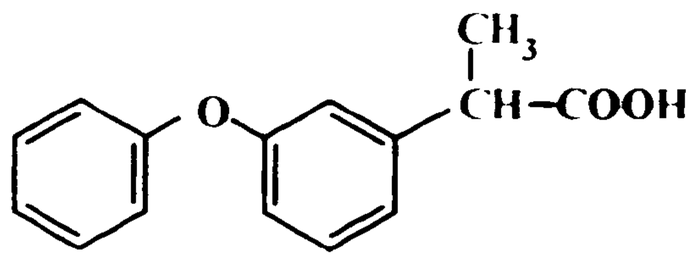
The non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of a wide variety of joint and musculoskeletal disorders, especially rheumatoid and osteoarthritis. As a group, the NSAIDs are structurally diverse, and includes the salicylates, pyrazoles, oxicams, fenamates, arylacetic acids and arylpropionic acids. Of these, the 2-arylpropionic acids (2-APAs) are amongst the most commonly used agents and have been the backbone of anti-inflammatory drug treatment since the 1970's, when the prototype, ibuprofen was marketed (Williams *et al.*, 1993). Since then a large number of analogues have been developed, the structures of some of which are shown in Figure 1.18.

1.8.1 Chemistry

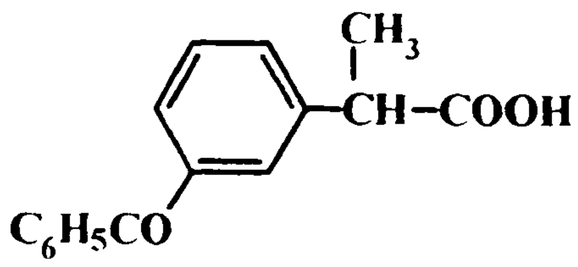
The 2-arylpropionic acids (profens) have three common structural components which are required for antiinflammatory activity. These include a carboxylic acid group, a substituted aromatic system at the α– carbon atom and the stereochemical configuration at the α - carbon must be *S*-. Being organic acids, these compounds are weakly acidic with pKa values between 4 - 5 (Albert and Serjeant., 1984), practically insoluble in water but soluble in organic solvents and alkaline solutions. The most widely studied drug of this group is ibuprofen (Figure 1.19). The majority of these agents are used as racemates, with the exception of (*S*)-naproxen and (*S*)-ibuprofen in Austria.



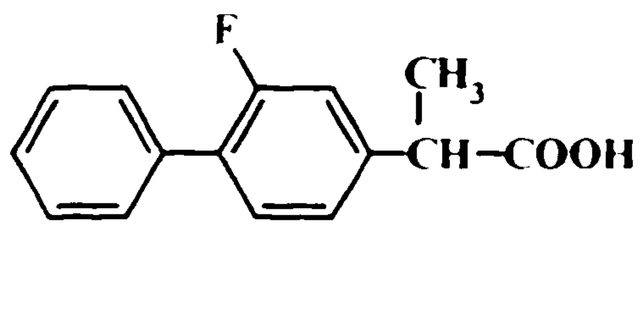
Ibuprofen



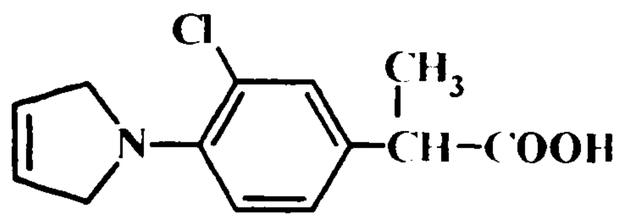
Fenoprofen



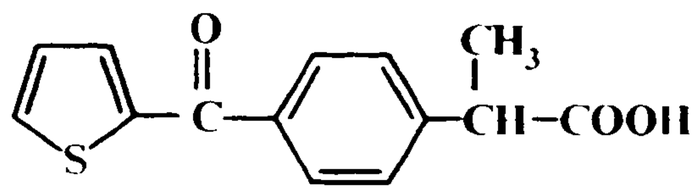
Ketoprofen



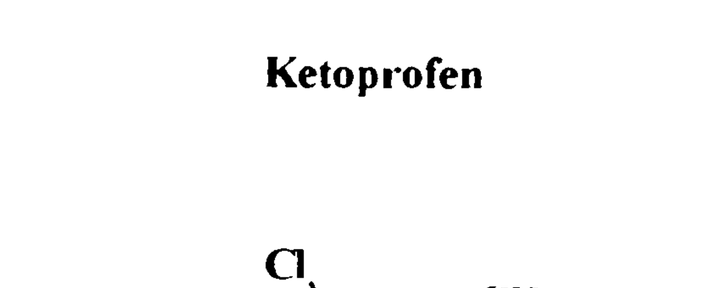
Flurbiprofen



Pirprofen



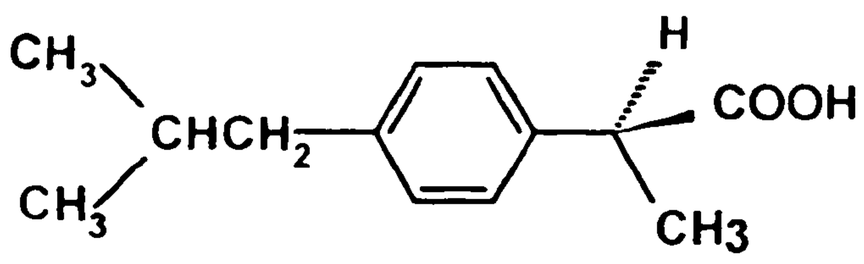
Suprofen



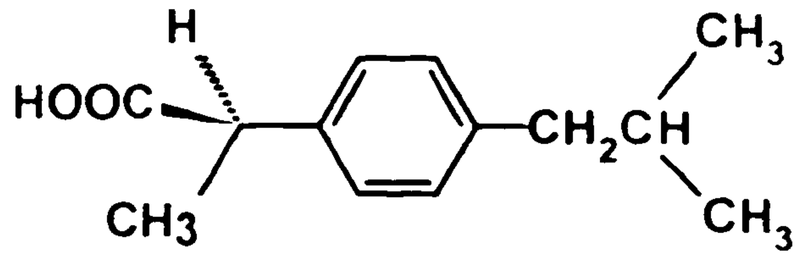
Carprofen

Naproxen

Figure 1.18: Structures of some 2-APA analogues.



(+)-(*S*)-Ibuprofen



(-)-(*R*)-Ibuprofen

Figure 1.19: Structures of ibuprofen enantiomers.

1.8.2 Pharmacology

a) Absorption

Absorption of the 2-APAs through the gastric mucosa is by passive diffusion and the oral bioavailability is generally very high. For example, the bioavailability of racemic ibuprofen has been estimated to be approximately 0.95 and that for (*R*)-ibuprofen is 0.92, indicating almost complete bioavailability for both enantiomers (Hall *et al.*, 1993).

b) Distribution

The profens are highly plasma protein bound (>99%). In most cases the binding is stereoselective, the nature of which depends on the particular drug. For ibuprofen, indoprofen and 2-phenylpropionic acid, plasma protein binding is greater for the *R*- enantiomer (Lapicque *et al.*, 1993). The reverse is true for flurbiprofen and carprofen while conflicting results have been reported for ketoprofen (Hayball *et al.*, 1991; Dubois *et al.*, 1993). Part of this problem of interpreting plasma protein binding data is the wide variety of methods and conditions used, the complexity of the situation is increased as the binding characteristics for some of these agents are dose and concentration dependent and there is mutually competitive displacement of protein binding by the drug enantiomers (Evans *et al.*, 1990). The apparent volume of distribution of these agents is relatively small, between 0.10 to 0.15 L/kg, indicating that tissue binding is less important than plasma protein binding (Lin *et al.*, 1987). Distribution into the synovium appears to be by passive diffusion, being influenced by the physicochemical properties of the drug as well as the pathological condition of the synovial membrane (Netter *et al.*, 1989). For ibuprofen and naproxen enantiomers, the concentration profiles in synovial fluid is dampened and sustained compared to the plasma profiles as a result of slow equilibrium, and generally reflects the stereochemical composition of the enantiomers in plasma. (Day *et al.*, 1988b; Seideman *et al.*, 1994). For ketoprofen and tiaprofenic acid, the synovial fluid contains equal enantiomer concentrations, consistent with the lack of

enantioselectivity in their plasma pharmacokinetics in man (Singh *et al.*, 1986; Foster *et al.*, 1989; Evans *et al.*, 1992).

c) Mechanism of action

Inflammation in tissue is caused by the action of cyclo-oxygenase (prostaglandin synthetase) and lipoxygenase enzymes on arachidonic acid. Prostaglandin synthetase activity leads to formation of prostaglandin D_1, E and F, prostacyclin and thromboxanes. Prostaglandins, especially prostaglandin E produces the features of inflammation. The action of lipoxygenase enzymes produces the leukotrienes, which also play a significant role in the inflammation process (Hart and Huskisson, 1984). The 2-APAs and other non-steroidal anti-inflammatory drugs (NSAIDs) act predominantly by inhibiting prostaglandin synthesis (Vane, 1971; Fereira and Vane, 1979). The *in-vitro* cyclo-oxygenase inhibitory activity of the profens resides principally in the enantiomers of the *S*-configuration, as reflected by the high eudismic ratios. Comparison of the relative potencies of the enantiomers in *in-vivo* test systems however show that the large eudismic ratios observed *in-vitro* are diminished such that the activity ratios for ibuprofen and fenoprofen are almost equal for both enantiomers (Table 1.1). This difference is due to the fact that chiral inversion occurs *in-vivo*, involving the biotransformation of the relatively inactive *R*-enantiomers to their pharmacologically active *S*-antipodes. Indeed, a large difference in the eudismic ratios *in-vivo* compared to that *in-vitro* is a good indication that metabolic chiral inversion has taken place (Adams *et al.*, 1976).

Table 1.1 Relative enantiomeric *in-vitro* and *in-vivo* activities of some 2-APAs (adapted from Hutt and Caldwell, 1984)

Compound	Eudismic Ratio (*S R*)		Test System
	in-vitro	*in-vivo*	
Carprofen	>23		PG synthetase inhibition
		13.5	Acute adjuvant arthritis (rat)
Fenoprofen	35	~ 1	PG synthetase inhibition (human)
Ibuprofen	160		PG synthetase inhibition (bovine)
		1.4	Pain threshold (rat)
		1.1	UV erythema (guinea pig)
Indoprofen	~100		PG synthetase inhibition
		18.3	Carrageenin induced oedema (rat)
Naproxen	133		PG synthetase inhibition (sheep)
		27.5	Rat paw oedema
		14.7	Antipyretic activity (rat)

Recently two distinct forms of cyclo-oxygenases, COX-1 and COX-2, encoded by two different genes have been discovered (Vane, 1994). COX-1 is found normally in most tissues and is responsible for cellular homeostasis, i.e. normal "housekeeping" functions such as maintaining the microvascular integrity of the GIT. COX-2 is only produced in inflammatory cells as a result of inflammation and tissue trauma. Therefore the prostaglandins derived from COX-2 activation are likely to be responsible for the features of inflammation and tissue damage (Dray and Bevan, 1993). Thus, development of anti-inflammatory agents with selective COX-2 inhibition would be a logical step in order to avoid the gastrointestinal side effects associated with these drugs. Indeed preliminary information has shown that agents with less inhibitory activity for COX-1, such as naproxen and indomethacin are less likely to cause GIT adverse effects than piroxicam, which is a more selective COX-1 inhibitor (Hayllar and Bjarnason, 1995). However, this issue is yet to be fully resolved in view of the multifactorial nature of the pathophysiology of

NSAID -induced adverse effects, and also the fact that *in-vivo* data are affected by other factors such as enantioselective pharmacokinetics.

In-vitro experiments have shown that the 2-APAs also inhibit lipoxygenase metabolism. However, the concentrations that are required are not likely to be achieved at the unbound concentrations present after normal therapeutic doses (Williams *et al.*, 1993). It has also been observed that the NSAIDs elicit pharmacological effects that are independent of cyclo-oxygenase inhibition. These include the ability to reduce sulphated glycosaminoglycan synthesis in articular cartilage *in-vitro*, suppression of neutrophil aggregation, degranulation and superoxide generation, inhibition of inflammatory oedema by action on polymorphonuclear leukocytes (Evans, 1992). It has been suggested that these prostaglandin-independent effects arise from the ability of the NSAIDs to partition into biological membranes, where they disrupt intercellular signalling events and subcellular protein-protein interactions. As such activities are based on partitioning behaviour, it is likely to be non-stereoselective (Evans,1992). This is supported by a recent report that of the equipotent *in-vitro* inhibition of human polymorphonuclear cell function by (*R*)-, (*S*)- and (*RS*)-ibuprofen (Villaneuva *et al.*, 1993).

1.8.3 Metabolic chiral inversion

A unique characteristic of the 2-APAs is their ability to undergo chiral inversion from the less active *R*- enantiomer to their more active *S*-antipodes (Caldwell *et al.*, 1988b). The extent of the inversion reaction is substrate and species dependent (Hutt and Caldwell, 1983). The generally accepted mechanism of inversion is represented in Figure 1.20 for ibuprofen. This involves reaction of (*R*)-ibuprofen with coenzyme A (CoA) to form the corresponding thioester. This thioester may undergo hydrolysis to regenerate the *R*- enantiomer, or epimerization of the profen moiety to yield the (*S*)-ibuprofen-CoA thioester via the formation of an acid enolate tautomer (Sanins *et al.*, 1991). The (*S*)-ibuprofen-CoA thioester

subsequently undergoes hydrolysis to yield (S)-ibuprofen, thus completing the inversion reaction (Nakamura *et al.*, 1981). The mechanistic aspects of the

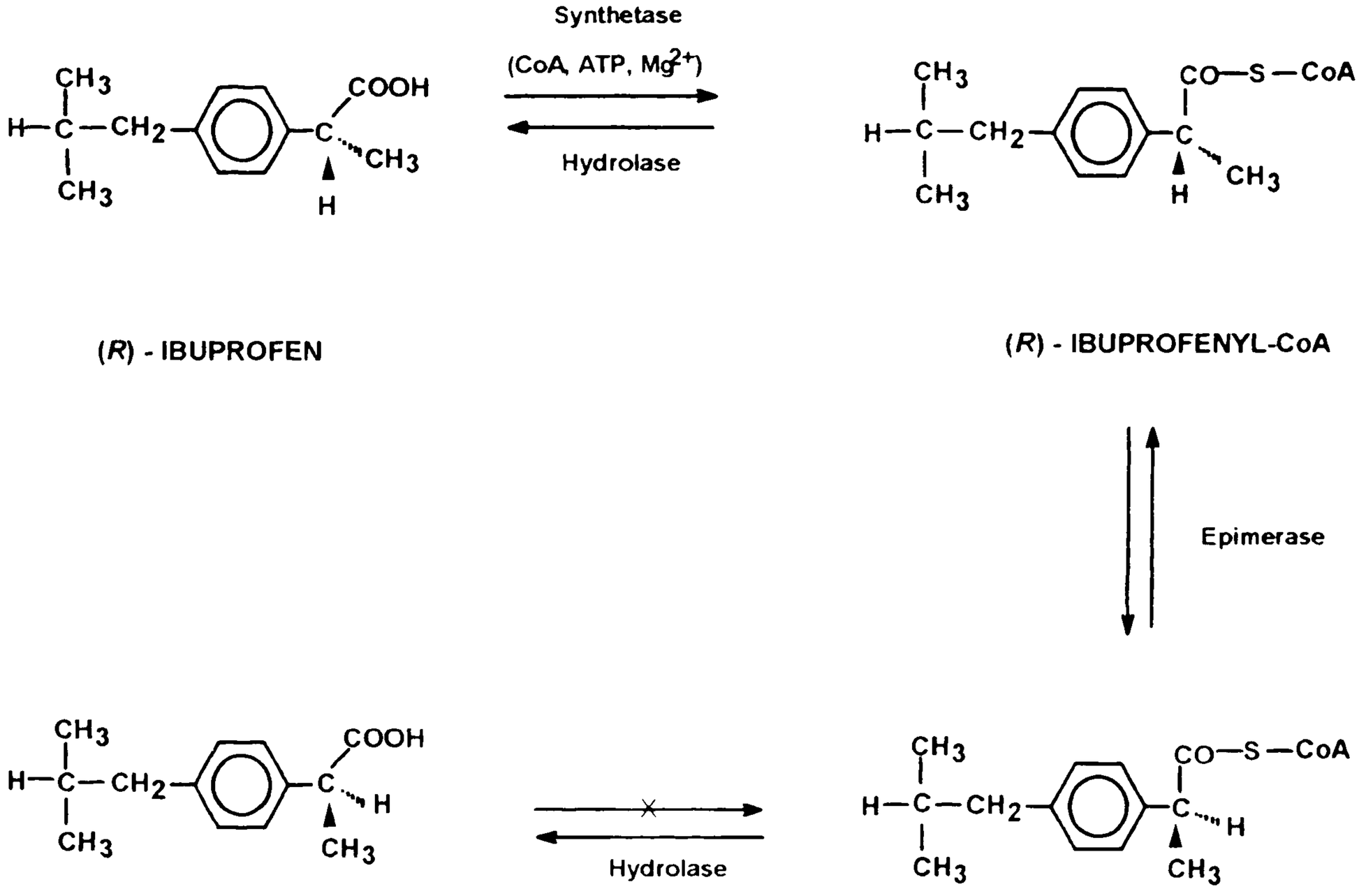


Figure 1.20: Schematic representation of the chiral inversion of ibuprofen (adapted from Tracy and Hall, 1991).

inversion reaction have been extensively investigated *in-vitro* using tissue preparations (Knihinicki *et al.*, 1989; Knadler and Hall, 1990; Knihinicki *et al.*, 1991; Tracy *et al.*, 1993), hepatocytes (Muller *et al.*, 1990) and perfused liver preparations (Nakamura *et al.*, 1981). The unidirectional nature of the inversion is due to the stereospecific formation of the CoA-thioester with (*R*)- ibuprofen but not (*S*)-ibuprofen (Knihinicki *et al.*, 1989) and appears to be mediated by long chain fatty acid synthetases (Tracy *et al.*, 1993). Recent evidence suggests that the formation of (*R*)-ibuprofen-adenylate is the rate limiting step to this reaction (Menzel *et al.*, 1994). The epimerization reaction appears to be non-stereoselective as synthetic samples of both diastereoisomeric thioesters yield the alternative isomer

(Tracy and Hall, 1991; Knihinicki *et al*., 1991). The reaction was originally thought to be spontaneous in nature but current evidence suggests it to be enzyme mediated (Mayer *et al*., 1988; Chen *et al*., 1990). An epimerase enzyme has recently been isolated and purified but its actual physiological function is as yet unknown (Shieh and Chen, 1993). From these *in-vitro* experiments, it generally accepted that inversion occurs mainly in the liver, although inversion has been observed in kidney preparations (Knihinicki *et al*., 1989). It has also been suggested that intestinal pre-systemic inversion may occur from evidence provided by *in-vitro* experiments using animal tissue preparations (Simmonds *et al*., 1980; Berry and Jamali, 1991; Jeffery *et al*., 1991; Sattari and Jamali, 1994) and indirect evidence in man (Jamali *et al*., 1988; Mevhar and Jamali, 1988). However, direct comparison of the extent of inversion after oral and intravenous dosing of ibuprofen in man did not show any significant differences (Hall *et al*., 1993), thus refuting the view put forward by Jamali and co-workers. Chiral inversion has also been demonstrated for ibuprofen using the isolated perfused lung preparations (Hall *et al*., 1992) but is unlikely to be significant *in-vivo* due to the large extent of protein binding.

The acyl-CoA thioester is a highly reactive species and is the intermediate form of all fatty acids prior to lipid synthesis or catabolism e.g incorporation of fatty acids into triglycerides, transport of long chain fatty acids across membranes, and for the β-oxidation of fatty acids. Thus, the formation of the (*R*)-2-APA-CoA thioesters is thought to have toxicological consequences. Fears *et al*., (1978) have demonstrated that fenoprofen, ibuprofen and ketoprofen following administration to rats could displace endogenous fatty acids and form hybrid triglycerides. On administration of (*R*)-ibuprofen and racemic ibuprofen to rats, both enantiomers were found on hydrolysis of triacylglycerols isolated from perinephric fat, but when (*S*)-ibuprofen was administered, negligible amounts of the drug were recovered, in fat (Williams *et al*., 1986). Thus, the formation of the thioester intermediates by the 2-APAs also paved a way for their involvement in fatty acid metabolism. It was postulated that in this manner the 2-APAs may perturb normal fatty acid synthesis and metabolism. It has also been suggested that these thioesters may acylate the amino groups of proteins resulting in formation of immunogens (Williams *et al*.,

1993). Thus physiological or pathological conditions that increase exposure to the thioester may enhance toxicity e.g. enhancement of 2-APA-CoA thioester formation by clofibrate (Knights *et al.*, 1991). These hypothesised consequences have yet to be conclusively proven to date, and it is worthwhile noting that there is no evidence as yet to prove that naproxen (marketed and used as the single S-enantiomer) is less toxic than the other 2-APAs administered as racemates (Williams and Day, 1988).

1.8.4 Other metabolic reactions

In addition to chiral inversion, the 2-APAs also undergo metabolic oxidation and conjugation reactions with amino acids and glucuronic acid. Glucuronidation is the predominant metabolic route for most of the 2-APAs, although functional oxidation is also important with some congeners. For example, ibuprofen is oxidised to form two major metabolites, hydroxy and carboxyibuprofen in man, forming 26 and 35 % of administered dose excreted in urine (Figure 1.21; Mills *et al.*, 1973). Irrespective of the nature of the form administered i.e. whether racemate or individual enantiomers, the optical rotation of both metabolites recovered in urine is dextrorotatory indicating possible stereoselectivity in the oxidation reaction. Interpretation of these results are complicated by the fact that the observation may be a result of extensive chiral inversion of ibuprofen and possibly of the metabolites (Caldwell *et al.*, 1988). With carboxyibuprofen, a second chiral centre is introduced, and thus it can exist as four stereoisomeric forms. Interpretation of the data to date is hampered by the methodological problems, i.e. the lack of resolution of all the four stereoisomeric forms (Kaiser *et al.*, 1976) and the assignment of the absolute configuration to the chromatographic peaks.

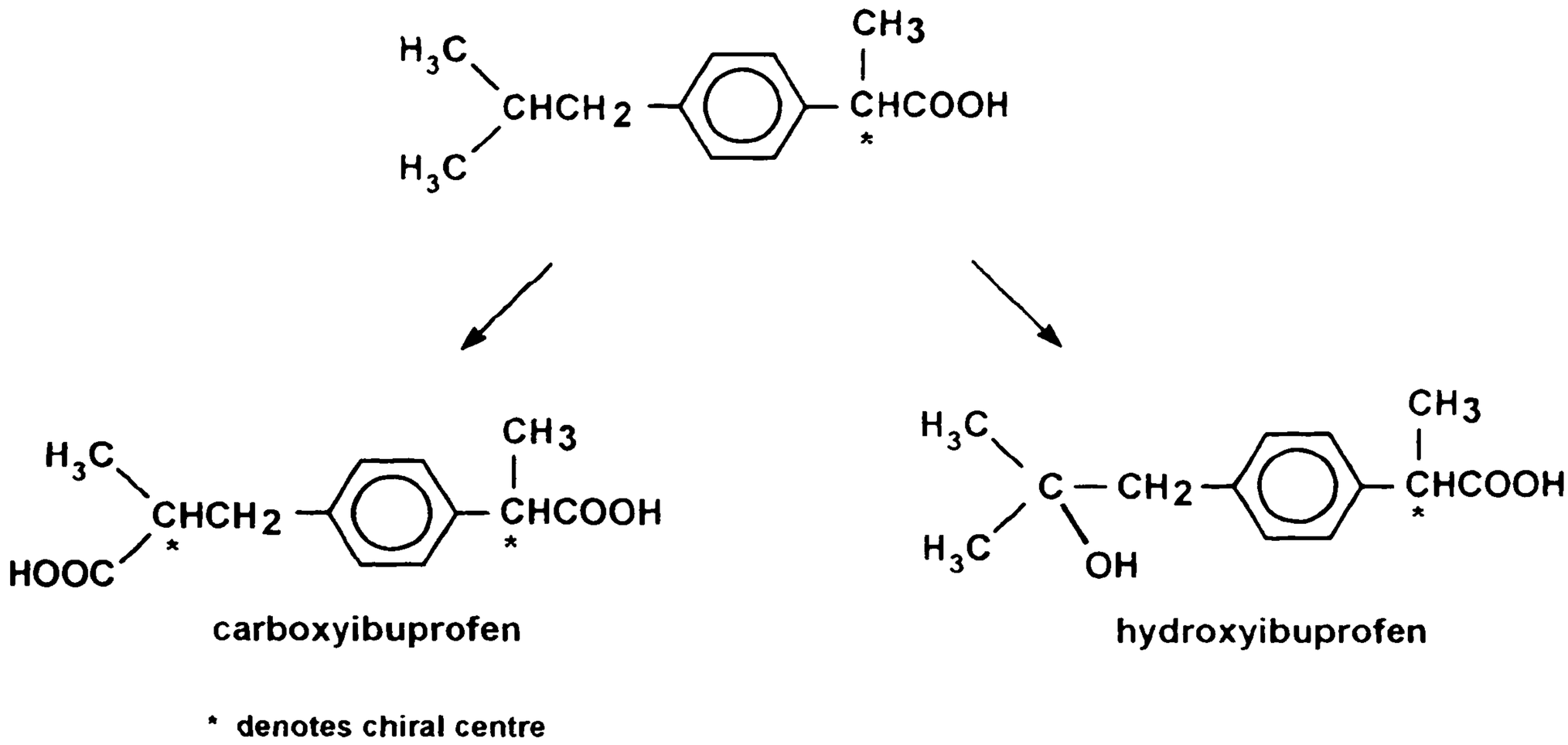


Figure 1.21: Metabolism of ibuprofen to yield the two major oxidation products hydroxyibuprofen and carboxyibuprofen.

In man, fenoprofen undergoes aromatic oxidation to form 4'-hydroxyfenoprofen which is subsequently excreted as the glucuronide. The majority of the metabolite recovered in urine has the *S*-configuration. As with ibuprofen, the stereoselectivity of the oxidation reaction is confounded by chiral inversion., which is extremely rapid in man (Rubin *et al.*, 1985). Flurbiprofen, which does not undergo chiral inversion in man, also undergoes aromatic hydroxylation to form the 4'-hydroxy and the 3',4'-dihydroxy metabolites, 4'-hydroxyflurbiprofen being the major metabolite. The 3',4'-dihydroxy metabolite undergoes further methylation to form the 3'-hydroxy-4'-methoxy product (Davies, 1995). Mean urinary recovery studies of (*S*)- and (*R*)-flurbiprofen and the 4'-hydroxy-metabolite reveal *S*/*R* ratios of 0.89 and 0.80 respectively, suggesting a lack of stereoselectivity of the oxidation reaction (Small *et al.*, 1990). However, another investigation revealed that the major urinary flurbiprofen metabolites had an *S*/*R* ratio of 0.80, which prompted the authors to suggest that enantioselectivity exists in the oxidation and conjugative reactions (Knadler and Hall, 1989). Several other NSAIDs also undergo oxidative metabolism e.g. loxaprofen, indoprofen, pirprofen and cicloprofen (Hucker *et al.*, 1980).

The 2-APAs and their principal metabolites undergo varying degrees of phase II conjugation reaction with glucuronic acid. The acyl glucuronides formed are relatively unstable and under physiological conditions may hydrolyse and release the parent molecule into the systemic circulation, a process termed futile recycling (Meffin *et al.*, 1983). With a decrease in renal function or renal impairment, accumulation of these glucuronides occurs and the effect of futile recycling is an increased plasma accumulation of these drugs (Meffin *et al.*, 1986). This problem is of great importance with elderly patients who frequently require these drugs (Williams *et al.*, 1993). The acyl glucuronides may also undergo acyl migration to yield positional isomers which are resistant to enzymatic hydrolysis by β-glucuronidase (Sinclair and Caldwell, 1981).

As glucuronic acid itself is chiral, the acyl glucuronides of the 2-APAs are diastereomers and as such, stereoselectivity may exist in their synthesis, renal clearance or hydrolysis. Evidence from *in-vitro* experiments have provided evidence that clearance by glucuronidation is indeed stereoselective (Caldwell *et al.*, 1988). With microsomes from rabbit, rhesus monkeys and human livers, enantioselectivity was evident in the glucuronidation of ibuprofen, benoxaprofen and naproxen, favouring the *S*- enantiomer in each case (El-Mouelhi *et al.*, 1987). In the case of ibuprofen, partial metabolic clearance by glucuronidation in humans was higher for the *S*- enantiomer (Lee *et al.*, 1985), indicating stereoselectivity in either one or more than one of the processes of formation, hydrolysis and renal clearance of the acyl glucuronide.

The acyl glucuronides are often considered to be reactive metabolites as they can irreversibly bind to protein macromolecules via covalent bonding (Hayball *et al.*, 1995; Stogniew and Fenselau, 1982; Hyneck *et al.*, 1988), forming complexes which are potentially immunogenic and suspected to cause anaphylactic reactions (Spahn-Langguth and Benet, 1992). Formation of such complexes have been observed *in-vitro* for benoxaprofen (Van Breenan and Fenselau, 1985), ketoprofen (Hayball *et al.*, 1992; Dubois *et al.*, 1994), carprofen (Iwakawa *et al.*, 1990) and fenoprofen (Volland *et al.*, 1991), and *in-vivo* with fenoprofen (Volland *et al.*,

1991). Thus, the profens that are principally cleared via glucuronidation e.g. ketoprofen, are potentially more likely to cause immunogenic responses in patients with impaired renal function. However, these toxic effects have yet to be conclusively proven and this is an area that deserves further investigation.

Many xenobiotics containing the carboxylic acid group are metabolised to conjugates with amino acids, principally glycine, glutamine and taurine (Hutt and Caldwell, 1990). The mechanism of this conjugation is thought to proceed via the formation of respective CoA thioesters. It is thus reasonable to expect that the 2-APAs that undergo extensive chiral inversion may also form amino acid conjugates. In dogs, glycine conjugates of 2-phenylpropionic acid (Tanaka *et al.*, 1992) and taurine conjugates of loxoprofen (Tanaka *et al.*, 1983) have been reported. In man taurine conjugates of (*R*)- and (*S*)-ibuprofen have recently been discovered accounting for about 1.5 % of the dose (Shirley *et al.*, 1994), a glycine conjugate was however not detected.

1.8.5 Renal excretion

Generally, renal excretion of unchanged 2-APAs is of little importance and accounts for small percentage of eliminated drug, reflecting their high lipophilicity (Williams *et al.*, 1993). Studies reporting significant excretion of unchanged drug are probably due to the hydrolysis of the labile acyl glucuronides in the bladder or *ex-vivo*. (Upton *et al.*, 1984; Faed, 1984).

1.9 Aims and objectives of the present investigation

As is apparent from the above discussion, the enantioselective pharmacokinetics and disposition of the 2-APAs are complicated by the following:

i) they are highly and enantioselectively bound to plasma proteins, which in the case of some of the analogues appears to be concentration and dose dependent, and give rise to enantiomer-enantiomer interactions;

ii) many of these agents undergo metabolic chiral inversion from the inactive *R*-enantiomers to the active *S*- antipodes in man;

iii) they also undergo stereoselective oxidation and conjugation reactions.

Various aspects of the stereoselective disposition of the 2-APAs have been subject to much research interest in an effort to understand the mechanisms involved and their relationship to one another, and on the influence of physiological and pathological conditions. These studies are quite varied with regards to experimental design and are often limited in usefulness as they address only specific aspects of the whole dispositional process. Other than naproxen, the profens are marketed and administered as racemates, and because of the occurrence of enantiomer-enantiomer interactions, studies involving administration of the racemic drug are important as they are a closer representation of the actual therapeutic situation. It is also important that unbound drug enantiomer concentrations are determined as plasma protein binding plays such a central role in influencing the disposition of these agents, and without which the interpretation of the enantioselectivity in hepatic and renal clearance mechanisms is impossible.

(*R,S*)- Ibuprofen is an important NSAID of the 2-APA group widely used in the treatment of a variety of inflammatory disorders and has recently become widely available in the U.K. as a result of a change in its legal status. As a result of the interest in the stereochemical aspects of the disposition of the 2-APAs, ibuprofen disposition has been of extensively investigated both *in-vivo* and *in-vitro* (Caldwell *et al.*, 1988; Williams *et al.*, 1993; Evans, 1992). However, few of the *in-vivo* investigations have presented a total picture of ibuprofen disposition following the administration of the racemate as a result of methodological difficulties associated with the determination of the enantiomeric composition of the free drug in plasma

and the determination of the stereochemical composition of the two major metabolites in urine (Kaiser *et al.*, 1976; Baillie *et al.*, 1989; Evans *et al.*, 1990; Rudy *et al.*, 1990). An additional problem with the data presented in the literature is that the majority of the studies are carried out on relatively young healthy volunteers, only during the course of these investigations described in this thesis have reports appeared which examine the effect of disease state on enantiomeric disposition (Li *et al.*, 1993; Chen and Chen, 1994). The pharmacokinetics of the drug in the intended target population should also be thoroughly studied. Epidemiological studies have shown that the elderly are the most frequent users of the NSAIDs and the most at risk of adverse effects (Day *et al.*, 1988a; Carson and Willet, 1993). To date only a few studies have focused on the effects of age on the disposition of the 2-APAs (Advenier *et al.*, 1983; Upton *et al.*, 1984).

The main objectives of the present investigation are to examine the enantiomeric disposition and pharmacokinetics of ibuprofen following the administration of the racemate to healthy young volunteers and to compare the data obtained with a similar study carried out in healthy elderly subjects. The aim being to establish if age has an influence on the stereoselective disposition of the drug as has been reported for other chiral drugs administered as racemates (Chandler *et al.*, 1988; Hooper and Qing, 1990).

In order to present a complete study on the stereoselectivity of ibuprofen disposition, it is intended to develop stereospecific analytical methodologies for the determination of both unbound and total drug in serum and also the free and conjugated drug in urine. In addition, relatively few methods have been published which describe the analysis of the hydroxy and carboxy metabolites of ibuprofen in urine and the determination of the stereochemical composition of the carboxy metabolites. The stereochemical composition of carboxyibuprofen following the administration of the racemic drug or *R*- ibuprofen has to date been problematical (Kaiser *et al.*, 1976; Young *et al.*, 1986; Baillie *et al.*, 1989; Rudy *et al.*, 1990). It is therefore intended to examine appropriate methodologies for the determination of total metabolite content in urine following administration of the racemic drug and

also their stereochemical composition. As authentic reference samples of the four stereoisomers of carboxyibuprofen are not available, an attempt will be made to isolate the single isomers and determine their absolute configuration using chiroptical methods.

CHAPTER 2

Stereospecific Analysis of Ibuprofen Enantiomers in Serum and Urine

2.1 Introduction

The recent surge in interest in the actions and disposition of drug enantiomers has been due, in part, to the advances made in analytical techniques (Hutt, 1990). The separation and analysis of enantiomers pose an additional challenge over conventional drug analysis as enantiomers have almost identical physicochemical properties, such as melting and boiling points, lipid solubility and chromatographic mobility. However, when placed in a suitable chiral environment such as plane polarised light or the presence of another chiral compound, differences can be discerned. Some of the more common techniques for enantiomeric analysis are presented in Table 2.1 below:

Table 2.1 Common enantiospecific analytical methodologies

Physical Methods	
	polarimetry, circular dichroism
	pseudoracemic mixtures of enantiomers labelled with either stable or radio isotopes
	NMR spectroscopy in the presence of chiral shift reagents, solvating or derivatising reagents
Separation Science Methods	
	chromatographic techniques
	capillary electrophoresis
Biological Methods	
	enantiospecific radioimmunoassay
	radioreceptor assays

(Adapted from Hutt *et al.*, 1994)

Bioanalysis involves analysing small quantities of analytes in a complex matrix, the exact composition of which is often unknown. Enantiospecific bioanalysis adds another dimension of difficulty as the biological media is invariably chiral in nature (Camillieri *et al.*, 1994). Therefore, it is not surprising that chromatographic techniques are most commonly used as these can distinguish between the individual enantiomers and the matrix, while providing excellent sensitivity to quantitate trace concentrations. As such the relevance of these techniques will be reviewed below.

2.1.1 Chromatographic resolution of enantiomers

Two major approaches are available in enantiospecific chromatography, the indirect and direct approach, each of which will be briefly examined.

a) Indirect approach

Using this approach, enantiomers are derivatized with optically pure chiral derivatizing reagents (CDAs) to form stable covalent diastereomers. As a result of their different physical and chemical properties, such diastereomers can be separated by conventional chromatographic techniques. The advantage of this approach is that achiral stationary phases are used and these are often more robust, stable, efficient and economical than chiral stationary phases (CSPs). In addition the derivatization reaction provides an opportunity to increase detection sensitivity by coupling enantiomers with suitable chromophores. However, this apparently simple approach has its pitfalls and these are summarised in Table 2.2 .

Table 2.2 Potential problems of indirect enantiomeric analysis

Enantiomeric purity of the CDA
Racemization of the CDA or analyte during derivatization
Stereoselective derivatization
Differential detector responses to the two diastereomers

(Adapted from Hutt *et al.*, 1994)

Of utmost importance is a knowledge of the purity of the CDA used. Only if the CDA is 100% optically pure is the diastereomer ratio representative of the enantiomeric composition in the original analytical sample. Otherwise, the optical purity of the CDA has to be taken into consideration to avoid errors in the determined values. This problem has been examined in greater detail by Allenmark (1988), Wozniak *et al.*, (1991) and Hutt *et al.*, (1994).

b) Direct approach

In the direct approach, enantiomers are separated on chiral stationary phases (CSPs) by *in situ* formation of diastereomeric complexes. For chiral recognition to occur, it has been proposed that a minimum of three complementary interactions, one of which must be sterically determined, are required between an analyte enantiomer and the CSP (Dalgleish , 1952), the antipode interacting at two sites only. Thus, the enantiomer that forms the more stable complex will be more strongly retained on the CSP and hence eluted later. The types of interactions involved depend on the CSP and may include hydrogen bonding, hydrophobic and dipole interaction, inclusion complex formation, charge transfer complexes and steric repulsion (Wainer, 1987). The interaction forces need not necessarily be attractive in nature. Repulsive interactions may also be involved in which case the enantiomer interacting at three points will be eluted first (Wainer, 1987; Hutt *et al.*, 1994).

One of the earliest reports of chiral separation involving CSPs were those of Danakov's on ligand exchange column chromatography (Armstrong, 1987). Since the first commercially available CSP for HPLC, developed by Pirkle *et al.*, (1981), the number of available CSPs has increased significantly. A classification system was suggested by Wainer (1987) based on their proposed modes of chiral recognition (Table 2.3). Recently multimodal CSPs , involving two or more modes of chiral recognition have been developed (Wallworth *et al.*, 1992).

The advantage of using the direct approach is that, in most cases, derivatization is not required. Where derivatization is required, the derivatizing reagent is usually achiral, and this avoids the problems of enantiomeric purity, racemization and differential detector responses that plague the indirect approach (Hutt, 1990).

Using the direct approach, quantitation of enantiomeric ratios and purity can be determined directly from peak areas and the quantitative properties are maintained as long as there is baseline resolution. However, in comparison with indirect methods, sensitivity is often poorer, due to peak asymmetry. Also, chromatographic selectivity is usually good between enantiomers but not so between enantiomers and achiral impurities. The choice of mobile phases which may be employed with CSPs are often limited and this may cause sample solubility problems. Last but not least, the presence of endogenous contaminants in bioanalysis, even in trace amounts may have marked effects on resolution and column stability (Wozniak *et al.*, 1991; Hutt *et al.*, 1994).

When using HPLC, chiral mobile phase additives may be also be used for enantiomeric resolution with achiral phases. The mechanism involved here is the formation of transient diastereomeric complexes between the analytes and additive. More commonly used additives used include the cyclodextrins and (+)-camphorsulphonic acid. The stability of the achiral phases and inexpensive additives are the main advantages of this approach. Chiral additives can be eluted and changed, and good selectivities can be achieved with the wide variety of

additives available. However, some of the additives have chromophores and thus impose a limitation on their usefulness (Armstrong,1987).

Table 2.3 Classification of chiral stationary phases

Type I:	Donor-acceptor CSPs
	examples: (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine
	(*R*)-*N*-(3,5-dinitrobenxoyl)valine
	(*S*)-*N*-(2-naphthyl)alanine
	interactions: hydrogen bonding, π –donor/acceptor, dipole-dipole stacking, steric
Type II:	Derivatised cellulose and amylose CSPs
	examples: synthetic ether,ester or carbamate derivatives of cellulose or amylose (e.g. Chiracel OD)
	interactions: hydrogen bonding, π –donor/acceptor, dipole-dipole stacking, partial inclusion complex formation
Type III:	Inclusion complex CSPs
	examples: α, β, γ-cyclodextrins
	interactions: hydrogen bonding, inclusion complex formation
Type IV:	Chiral ligand exchange CSPs
	examples: amino acids bound to silica via 3-glycycloxypropyl spacer group.
	interactions: reversible complex formation between metal ions and chiral complexing agents.
Type V:	Affinity or protein CSPs
	examples: α_1–acid glycoprotein, bovine/human serum albumin, ovomucoid
	interactions: hydrophobic, polar, steric.

(adapted from Wainer, 1987)

The use of HPLC techniques in chiral separations has overshadowed that of GC. The usefulness of GC is limited for a number of reasons. High column temperatures are usually involved and this may cause racemization of the chiral phase or analyte. When indirect methods are involved, apart from all the constraints discussed in section 2.1.1, the diastereomers formed must be sufficiently volatile. The stability differences between diastereomeric complexes may be very small at high temperatures, with subsequent poorer chromatographic resolution. Also, large scale preparative separations are generally not feasible in GC. Last but not least, there is a wider choice of stationary phases and mobile phase additives in LC (Allenmark, 1988; Armstrong, 1987).

Recent developments in electrophoresis and supercritical fluid chromatography have seen application in chiral separations (Lynam and Nicolas, 1993; Rawjee *et al.*, 1993; Terabe *et al.*, 1994). However, these techniques have yet to find wide acceptance in bioanalysis.

2.1.2 Enantiospecific analysis of ibuprofen and related 2-arylpropionic acids in biological fluids

Published methods for the enantiospecific analysis of the profens involve both direct and indirect approaches and in the majority of cases, these are based on HPLC methodologies.

a) Direct method of enantiomeric analysis of 2-arylpropionic acids

One of the first direct methods for the resolution of the enantiomers of a profen used a Pirkle type column with a (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine chiral selector (Wainer and Doyle, 1984). The acids were derivatized into both esters and amides forms and the best resolution was obtained with 1-naphthalenemethylamide derivatives. Based on these experiments, a model was proposed for the molecular interaction between the analyte derivatives and the CSP

to account for the elution order. The model involves the stacking of amide dipoles with supplementary $\pi - \pi$ interaction of the aromatic rings and hydrogen bonding. An alternative antiparallel interaction model was proposed, using a similar chiral phase in which the interactions between the analyte and the CSP are based on an antiparallel alignment of the amide dipoles with hydrogen bonding and $\pi - \pi$ interactions between the aromatic ring of the profen and the unsubsituted phenyl ring of the CSP, and $\pi - \pi$ interaction between the aromatic group of the amide and the dinitrophenyl ring of the CSP (Nicoll-Griffith, 1987). Subsequent work on flurbiprofen (McDaniel and Snider, 1987) seemed to support Wainer's hypothesis. New Pirkle type phases that were developed were used to separate ibuprofen enantiomers as 3,5-dinitroanilide derivatives (Pirkle *et al.*, 1986). The use of Pirkle type phases for the bioanalysis of the 2-APAs were published by Crowther *et al.*, (1984), Sioufi *et al.*, (1987) and Nicoll-Griffith *et al.*, (1988). In all these methods, derivatization was required before satisfactory resolution was obtained.

A CSP based on a human α_1-acid glycoprotein (AGP) was developed by Hermansson and Eriksson (1986). With the use of dimethyloctylamine as modifier in 2% isopropanol in phosphate buffer, enantiomeric separation of ibuprofen, ketoprofen and naproxen was achieved without prior derivatization. AGP columns have been used in pharmacokinetic and metabolic studies of flurbiprofen (Geisslinger *et al.*, 1992), naproxen (Anderson *et al.*, 1992) and ibuprofen (Geisslinger *et al.*, 1990; Petterson and Olsson, 1991). The main advantage of this CSP is that simple reversed phase solvents are used, and by varying the modifier and pH, good baseline resolution can be obtained. However, being based on biological proteins, wide batch to batch variations are observed in the stationary phases which limits their utility (Petersson and Olsson, 1991). Also, resolution and separation power decreases with increasing number of analyses of plasma samples i.e. the columns are not robust (Geisslinger *et al.*, 1992). Hutt and Caldwell (1988) reported separation of mandelic acid but not those of 2-arylpropionic acids on a commercially available bovine serum albumin column. Human serum albumin (HSA) protein phases used alone or mixed with AGP have had better success (Aubry *et al.*, 1994). Ovomucoid, avidin and flavoprotein columns have also been used

with varying success (Haginaka *et al.*, 1994; Mano *et al.*, 1994). Recently avidin (Oda *et al.*, 1991) and ovomucoid bonded phases (Haginaka *et al.*, 1993), that allow direct injection of serum, have been used for the enantioseparation of ketoprofen. These columns work on similar principles as the shielded hydrophobic phase columns. Proteins, especially glycoproteins have outer hydrophilic and inner hydrophobic surfaces. Large molecules such as serum proteins, would be eluted in the void volume, while smaller drug molecules can penetrate and interact with the shielded internal surface. These columns would be particularly useful for routine therapeutic monitoring of enantiomer concentrations should the need arise.

Apart from proteins, other naturally occurring molecules have also been used as chiral selectors, the most common of these being cellulose derivatives. Wainer (1987) has classified them as type II, and it is thought that these CSPs interact with the analyte through attractive interactions and form inclusion complexes. Stereoselectivity is based upon steric fit of the analyte into the chiral cavities. Okamoto *et al.*, (1989) reported the separation of flurbiprofen, tiaprofenic acid and ketoprofen on amylose tris(3,5-dimethylcarbamate) phases (Chiralpak™ AD) without derivatization. Ibuprofen enantiomers however, could only be resolved after derivatization into their methyl esters. The utility of these cellulose phases in bioanalysis was demonstrated by the stereospecific analysis of flurbiprofen in urine using a cellulose tris(4-methylbenzoate) (Chiralcel™ OJ) phase, after derivatization into their methyl esters (Aboul-Enein *et al.*, 1992). The main disadvantage of these phases is the limited choice of mobile phases that may be used, as they are incompatible with chlorinated and aqueous solvents.

Type III CSPs separate enantiomers based on the mechanism of inclusion complexation. They are usually synthetic polymers or biopolymers, the most common of these being β-cyclodextrin. They have been successfully used to resolve underivatized ibuprofen enantiomers following isolation from biological fluids (Geisslinger *et al.*, 1989). A simple aqueous-organic mobile phase was used but moderately elevated temperatures were necessary to improve the chromatography. Even then, the separation of the enantiomers was incomplete, with relatively long

retention times and significant batch to batch differences between the CSPs were also reported.

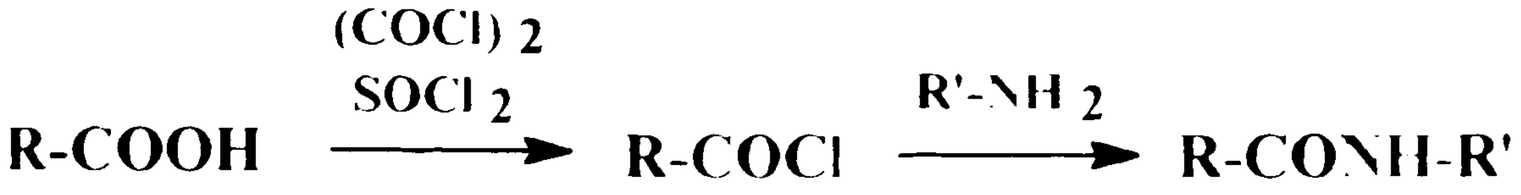
Chiral mobile phase additives have also been used in the separation of naproxen and other 2-APAs. Separation was achieved by use of cinchona alkaloids in the mobile phase (Petersson, 1984). Normal phase columns were used, the separation efficiency depended on the type of stationary phase i.e whether silica, diol or cyano bonded columns were used. The usefulness of this method is limited by the fact that these alkaloids are strong UV chromophores which would adversely affect analytical sensitivity. Hutt and Caldwell (1988) attempted to resolve 2-phenylpropionic acid, ibuprofen and pirprofen using β-cyclodextrin as a mobile phase additive but reported unsatisfactory results.

b) Indirect methods of enantiomeric analysis of 2-arylpropionic acids

For a drug to be analysed using indirect methods, it must possess a suitable functional group(s) that can be derivatized. The 2-arylpropionic acids possess a carboxyl group that can be easily transformed into an ester or amide moiety. Lee *et al.*, (1984) and Johnson *et al.*, (1979) used (S)-2-octanol to derivatize ibuprofen and naproxen to form their respective esters. This approach is generally not favoured due to the relative instability of esters (Testa, 1986) and the poorer resolution of esters compared to amides on silica and alumina columns. Generally, the 2-APAs are converted to amides by reaction with an optically pure amine prior to analysis by either normal or reversed phase chromatography.

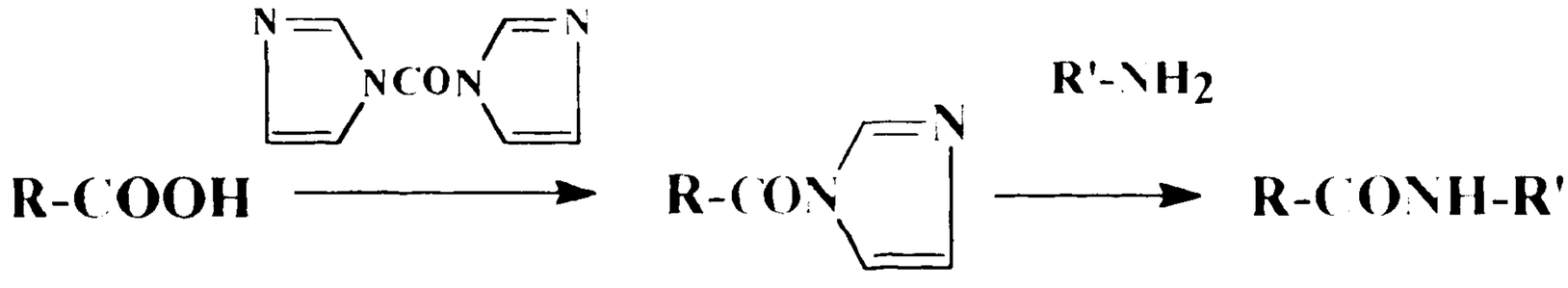
Derivatisation of the 2-APAs to amides is usually carried out by chemical activation of the carboxy group followed by reaction with a chiral amine. This chemical activation frequently involves one of four methods (Hutt, 1990):

a) formation of acyl chlorides using thionyl and oxalyl chlorides

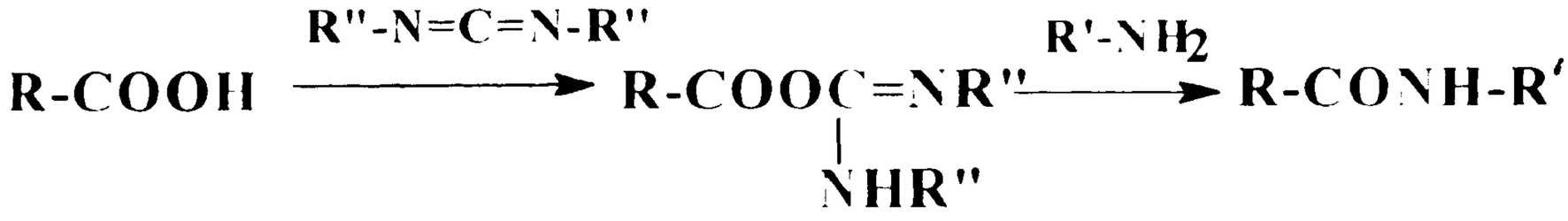


b) formation of mixed anhydrides using ethylchloroformate

c) reaction with 1,1'-carbonyldiimidazole to yield an imidazolide



d) reaction with a carbodiimide to yield an O-acyldialkylisourea derivative



Reaction with thionyl chloride, to convert the carbonyl group of the profens to the corresponding acyl chloride, followed by reaction with either (*R*)- or (*S*)-1- phenethylamine was used in the early GC assays of ibuprofen (Brooks *et al.*, 1974) and indoprofen (Tosolini *et al.*, 1974). Due to undesired side reactions (Kaiser *et al.*, 1976) this activating reagent has been largely abandoned in favour of others. Among the more commonly used coupling reagents is 1,1'-carbonyldiimidazole which converts the carboxyl group into the corresponding imidazolide derivative (Maitre *et al.*, 1984; Young *et al.*, 1989; Rudy *et al.*, 1990;). However, various problems have been reported including the formation of *N,N*-disubstituted urea derivatives and uncertainty over conditions and concentrations that are considered as optimal (Hutt, 1990). Initially developed by Bjorkman (1985) for use with L-leucinamide as CDA, ethylchloroformate in the presence of triethylamine (TEA), has been widely applied for coupling 2-arylpropionic acids to a variety of amines (Spahn, 1987; Palylyk and Jamali, 1991; Wright *et al.*, 1992; Lemko *et al.*, 1993). The reaction involves formation of a mixed anhydride which is subsequently reacted with an amine CDA to yield the required diastereoisomeric derivatives. The main attraction of this approach is a short reaction time of 2 - 3 minutes. For the derivatization of tiaprofenic acid, a more reactive analogue, 2,2,2-trichloroethylchloroformate was required. Subsequent studies however, revealed that racemization occurred with tiaprofenic acid (Hutt *et al.*, 1994), ibuprofen (Ahn *et al.*, 1994), ketoprofen and flurbiprofen (Wright and Jamali, 1993). The extent of the racemization appears to be influenced by the nature of the base (Hutt *et al.*, 1994), solvent (Ahn *et al.*, 1994) and the concentration of chloroformate used (Wright and Jamali, 1993). The fourth method of activation involves the use of a carbodiimide and has been used for pirprofen, carprofen and 2-phenylpropionic acid, (Hutt *et al.*, 1986), and ibuprofen (Avgerinos and Hutt, 1987). Although no problems of racemization have so far been reported, the major disadvantage of this approach is that long derivatization times of up to 2 hours are required (Hutt *et al.*, 1986).

A variety of chiral amines have been used as CDAs, the most common of which being L-leucinamide, (*R*)- and (*S*)-1-phenethylamine, (*R*)- and (*S*)-1-

1-yl)ethylamine, (*R*)- and (*S*)-amphetamine. These CDAs are easily available commercially in very optically pure forms. The use of phenethylamine affords the amide an extra chromophore, thus increasing assay sensitivity. With the use of (naphthen-1-yl)ethylamines, this effect would be enhanced, and in addition, fluorescence detection can be used (Lemko *et al.*, 1993). Other detection-enhancing CDAs such as 1-(4-dimethylaminonaphthen-1-yl)ethylamine (Goto *et al.*, 1980; Nagashima *et al.*, 1985), 1-(4-dansylaminopropyl)-N-(2-phenylpropionyl)ethylamine (Iwaki *et al.*, 1994) and 1-(2-anthryl)ethylamine (Goto *et al.*, 1986) have also been used and in the latter case, detection limits of 100 fmol was reported for naproxen. Unfortunately, these reagents are not commercially available, thus limiting their potential usefulness.

2.1.3 Sample preparation procedures

As with most bioanalytical procedures, sample clean-up steps are vitally important for the enantioseparation of the profens. As the carboxylic moiety of the profens has a pKa between 4 - 5 (Albert and Serjeant, 1984), the biological sample is usually acidified or buffered below these values and extracted using an organic solvent. Sample clean-up also affords the analyst a pre-concentration step whereby the analyte can be concentrated down to a small volume before injection. This increases the final sample concentration considerably, thereby increasing analytical sensitivity.

However, sample clean-up procedures in enantiomeric analysis may present additional problems. It has been shown that sample clean-up may alter enantiomeric ratios or result in enrichment of the sample that is present in excess. Thus, it is important that analytical procedures be appropriately validated to ensure that non-chiral differentiation does not occur (Hutt, 1990).

In order to study the stereoselective inversion as well as the stereoselectivity of the oxidation and glucuronidation of ibuprofen, proper enantiospecific analytical procedures must be developed for its determination in serum and urine. A

knowledge of the extent of protein binding of the enantiomers is also necessary in order to determine the stereoselectivity in intrinsic metabolic clearance. The following section describes the development and validation of an indirect HPLC procedure that fulfils these requirements.

2.2 Experimental

2.2.1 Chemicals and reagents

Acetonitrile, hexane and ethanol (HPLC) grade were purchased from Rathburn (Walkerburn, U.K.). Sodium dihydrogen phosphate, sodium hydroxide diethylether (Analar grade), other solvents (GPR grade) and Merck silica gel GF_{254} for TLC were obtained from BDH (Poole, Dorset, U.K.). Ketoprofen, naproxen, ethylchloroformate and triethylamine (TEA) were obtained from Sigma Chemicals (Poole, Dorset, U.K.). (*R*)-1-(naphthen-1-yl)ethylamine (*R*)-NEA), 1-(3-dimethyl aminopropyl)-3-ethylcarbodiimide hydrochloride (CDI) were obtained from Aldrich (Gillingham, Dorset, U.K.), 1-hydroxybenzotriazole (HOBT) was obtained from Fluka Chemicals (Poole, Dorset, U.K.). (*R,S*)-, (*S*)- , (*R*)-ibuprofen, and (*R,S*)-flurbiprofen were a gift from Boots Ltd. (Nottingham, U.K.).

2.2.2 Chromatographic columns and supplies

The C_{18} column (Waters Resolve C_{18}), 5 µm, 150 x 3.9 mm, was obtained from Anachem Ltd. (Luton, Beds, U.K.). Refillable guard columns (10 x 2.1 mm) were packed with pellicular C_{18} (40 - 63 µm), both obtained from Alltech Associates (Lancs, U.K.). Merck silica gel (40-63 µm) for preparative chromatography was obtained from BDH (Poole, Dorset, U.K.) and were used in solid phase extraction (SPE) cartridges. Empty Bond-elut SPE cartridges and frits were obtained from Anachem Ltd. (Luton, Beds).

2.2.3 Instrumentation

Reversed phase HPLC was performed using an LDC Constametric 3000 pump, LDC Spectromonitor 3100 UV detector, Merck Hitachi spectrofluorometric detector (Poole, Dorset, U.K.), and LDC C4100 computing integrator (Stone, Staffs.

U.K.). Samples were injected using a Perkin Elmer ISIS 100 autosampler (Beaconsfield, Bucks, U.K.) fitted with a 100 µl sample loop. Proton nuclear magnetic resonance spectra were recorded using a Perkin Elmer R-32 spectrometer (Beaconsfield, Buck, U.K.) Fluorescence spectra were measured using a Hitachi F1000 spectrofluorometer (Poole, Dorset, U.K.).

2.2.4 Synthesis of (*R*)-1-(naphthen-1-yl)ethylamides of (*R*)- and (*S*)-ibuprofen

(*R,S*) -Ibuprofen (0.97 mmol, 200 mg) was dissolved in acetonitrile (5 ml) and the solution was cooled in an ice-bath. To this was added 200 µl of TEA (1.47 mmol) and 140 µl (1.47 mmol) of ethylchloroformate. The mixture was gently shaken from time to time. After 15 minutes, 250 mg (1.47 mmol) of (*R*)-NEA was added and the mixture was left to react at room temperature. After 1 hour, the solution was evaporated to dryness in a rotary evaporator. The residue was redissolved in 20 ml of diethylether, which was then washed twice with 20 ml of 1.0M HCl. This was followed by two further washings with distilled water (20 ml). The ether layer was removed, dried with anhydrous sodium sulphate, filtered and then evaporated to dryness. The dry residue was then spotted on a preparative TLC plate (20 x 20 cm; 1 mm thickness) coated with silica gel GF_{254} and developed in a solvent of hexane:ethyl acetate (6:1, v/v). The solvent was allowed to migrate for 18 cm, after which the plate was dried and viewed under a UV light source. The two separate bands observed under the UV light and the silica between 6.5 to 7.2 cm and 10.7 to 11.2 cm were scraped off the plate and separately extracted with 20 ml diethylether. After centrifugation at 1000g for 5 minutes. the ether layer was separated, filtered through a filter paper and evaporated using a rotary evaporator. The crystals obtained were recrystallized twice from ethanol. The melting points, NMR and fluorescence spectra (dissolved in acetonitrile:0.01M phosphate buffer, pH 3.5; 50:50 v/v) were recorded. The fluorescence spectra are presented in Appendix 1.

(*R,R*)-Ibuprofen diastereomeric amide derivative: mpt., 125-127°C; NMR (90 MHz $CDCl_3$), δ.ppm: 0.84 (d, 6H, $CH(C\underline{H}_3)_2$), 1.46 (d, 3H, $C\underline{H}_3CHCO$), 1.54 (d, 3H,

$C\underline{H}_3CHNH$), 1.78 (m, 1H, $CH_2C\underline{H}(CH_3)_2$), 2.39 (d, 2H, $C\underline{H}_2CH(CH_3)_2$, 3.52 (q, 1H, $CH_3C\underline{H}CO$), 5.65 (br.d, 1H, CON$\underline{H}$), 5.70 (br.q, 1H, $CH_3C\underline{H}NH$), 7.08 (dd, 4H, para-subsituted phenyl $\underline{H}$), 7.2 - 8.0 (m, 7H, naphthyl $\underline{H}$); MS: (EI, 35eV) *m/z* (relative intensity %); 360 (21.3), 359 ($M.^+$ 61.5), 197 (14.9), 162 (22.1), 161 (88.3), 155 (100), 119 (13.2), 105 (5.4). Elemental analysis: Found (%); C, 83.86; H, 8.21; N, 3.86, required for $C_{25}H_{29}NO$; C, 83.51; H 8.14; N, 3.90.

(*R,S*)-Ibuprofen diastereomeric amide derivative: mpt., 125-126^0 C; NMR (90 MHz $CDCl_3$), δ.ppm; 0.85 (d, 6H, $CH(C\underline{H}_3)_2$), 1.45 (d, 3H, $C\underline{H}_3CHCO$), 1.47 (d, 3H, $C\underline{H}_3CHNH$), 1.81 (m, 1H, $CH_2C\underline{H}(CH_3)_2$), 2.41 (d, 2H, $C\underline{H}_2CH(CH_3)_2$, 3.43 (q, 1H, $CH_3C\underline{H}CO$), 5.67 (br.d, 1H, CON$\underline{H}$), 5.82 (br.q, 1H, $CH_3C\underline{H}NH$), 7.11 (dd, 4H, para-subsituted phenyl $\underline{H}$), 7.2 - 8.0 (m, 7H, naphthyl $\underline{H}$); MS: (EI, 35eV) *m/z* (relative intensity %); 360 (32.2), 359 ($M.^+$ 80.3), 198 (5.8), 197 (18.5), 162 (29.1), 161 (100), 156 (37.4), 155 (91.3), 154 (18.6), 153 (15.8), 127 (6.9), 119 (21.4), 118 (15.7), 117 (16.3), 105 (8.3), 91 (8.5).Elemental analysis: Found (%); C, 82.58; H, 8.67; N, 3.89, required for $C_{25}H_{29}NO$; C, 83.51; H, 8.14; N, 3.90.

2.2.5 Enantiospecific analysis of ibuprofen in serum

a) Extraction

To serum samples (0.5 ml) was added 1 µg (10 µl of a 0.1 mg/ml solution in acetonitrile) of (*R,S*)-flurbiprofen as internal standard. The samples were then acidified by the addition of HCl (1.0M; 100 µl) and then buffered to pH 3.8 with sodium phosphate buffer (1.0M;pH 3.8; 1.0 ml)). Diethylether (3 ml) was then added and the samples mixed on a test-tube rocker for 20 minutes. The samples were centrifuged at 1000g for 2 minutes and the supernatant diethylether layer was transferred into separate tubes and dried under nitrogen at 40^0 C using a dry block heater.

b) Derivatization

The derivatization procedure was based on the method of Avgerinos and Hutt (1987), with minor modifications. To the dried residues of sample extracts/standard solutions were added 100 µg each of CDI, HOBT and (*R*)-NEA (100 µl each of a 1 mg/ml solution in dichloromethane). The tubes were tightly capped and then briefly vortex mixed. The reaction was allowed to proceed for 2 hours at room temperature. SPE silica cartridges (100 mg) were activated by elution with methanol (1 ml) and subsequently dried in a hot air oven at 100^0 C for 1 hour. Before use the cartridges were equilibrated with dichloromethane (1 ml). The reaction mixtures were then passed through the cartridges and the eluates collected. The cartridges were further eluted twice with 1 ml dichloromethane:acetonitrile (9:1, v/v) and the eluate pooled. The pooled eluate were then dried under nitrogen gas at 40^0 C in a dry block heater and reconstituted with HPLC mobile phase (100 µl). All samples were analysed in duplicate.

In order to determine the reaction yield for the derivatization reaction under the conditions described above, (*R,S*)-ibuprofen (2 µg) was derivatised as before and subjected to chromatographic analysis. The results obtained were compared to those obtained from chromatographic analysis of equivalent molar amounts of the synthesized amide derivatives described in section 2.2.4.

c) Chromatographic conditions

The mobile phase used was phosphate buffer (pH 3.5, 0.01M):acetonitrile (50:50, v/v) run at a flow rate of 1.5 ml/min. The detector was set at excitation and emission wavelengths of 290 and 330 nm respectively. The column used was a Waters Resolve C_{18} (5 µm, 150 x 3.9 mm) protected by a guard column (10 x 2.1 mm) filled with pellicular C_{18}. Aliquots of 50 µl of reconstituted extracts/standards were injected into the HPLC. The elution order of the diastereomeric amides were

determined by derivatizing and analysing pure enantiomers of (*R*)- and (*S*)-ibuprofen and (*R*)- and (*S*)-flurbiprofen.

d) Validation of Analytical Method

A stock solution of (*R,S*)-ibuprofen (1 mg/10 ml) was prepared in acetonitrile. Into six separate 10 ml volumetric flasks were pipetted 0.02, 0.05, 0.1, 0.2, 1.0 and 2.0 ml of the stock solution. The solutions were evaporated gently under nitrogen gas. and drug free serum added to the q.s. 10 ml to give final solutions of 0.1, 0.25, 0.5, 1.0, 5 and 10 µg/ml of each enantiomer. Aliquots (0.5 ml) of these standards were transferred into different tubes and stored at -20^{0} C. On each day of analysis one set of these tubes (0.1 – 10 µg/ml) were analysed together with the samples. Calibration curves were constructed by plotting peak area ratios (ibuprofen enantiomer:(*S*)-flurbiprofen derivatives) against the concentration of each enantiomer and subjecting this data to linear regression analysis. The concentration of each enantiomer in serum samples were determined by comparing their respective peak area ratios to the calibration curve prepared.

The accuracy and within day variation of the assay was assessed by analysing six "spiked" serum samples with the following ibuprofen enantiomer concentrations: 0.1, 1.0 and 10 µg/ml. The precision and accuracy of the assay was determined for each enantiomer by calculation of the coefficient of variation (standard deviation/mean x 100%) and mean percentage difference (mean concentration - actual concentration / actual concentration x 100%). The recovery of the extraction procedure was determined by comparing peak areas obtained with direct injections of standard solutions of equivalent concentrations of the drug.

The between day variation of the assay was determined by analysing "spiked" serum samples of 0.1, 1.0 and 10 µg/ml enantiomer concentrations for six consecutive days. The coefficient of variation and accuracy were calculated as described above.

In order to determine whether the analytical procedure could produce accurate data with respect to enantiomeric composition over a wide range of concentrations, a series of "spiked" standards were prepared of varying concentration and enantiomeric composition from individual (*R*)- and (*S*)-ibuprofen standards. "Total" ibuprofen concentrations of 0.5, 2.0 and 10.0 µg/ml were prepared with the following enantiomeric compositions; *R*:*S*; 1:4; 2:3; 3:2 and 4:1 for each concentration. Each of the samples were analysed in triplicate and the precision and accuracy for each composition at each concentration level were determined described above.

2.2.6 Enantiospecific analysis of ibuprofen in urine

a) Extraction procedure

For the enantiomeric determination of unconjugated ibuprofen in urine, 0.5 ml urine samples were used. To the urine samples were added 1 µg of flurbiprofen (10 µl of 0.1 mg/ml solution in acetonitrile), 100 µl of HCl (1.0M) and sodium phosphate buffer (pH 3.8; 1.0M; 1.5 ml). Hexane:isopropanol (9:1, v/v; 5 ml) was added as extraction solvent. The tubes were then capped and mixed for 20 minutes on a test-tube rocker, followed by centrifugation at 1000g for 5 minutes. The supernatant organic layer was separated and transferred into separate tubes and dried under nitrogen gas at 40^{0}C on a dry block heater. The residues were reconstituted in HPLC mobile phase (100 µl) prior to analysis.

For the determination of conjugated enantiomer levels, a 0.1 ml urine sample was used. To the samples were added 1 µg of flurbiprofen as internal standard, followed by 20 µl of 1.0 NaOH. The hydrolysis reaction was left to proceed for 2 hours at room temperature, after which time HCl (1.0M, 40 µl), sodium phosphate buffer (pH 3.8; 1.0M; 200 µl) and hexane:isopropanol (9:1, v/v; 1.0 ml) was added. The samples were then extracted as before.

In order to determine whether the 2 hour hydrolysis reaction was sufficient to completely liberate all the acyl glucuronides, a 5 ml urine sample from a volunteer who was administered 400 mg of the racemic drug was hydrolysed with sodium hydroxide (1.0M, 1.0 ml), as described above. At 30 minutes intervals, a 0.1 ml sample was pipetted out and analysed as before.

b) Derivatization procedure and chromatographic procedures

These were identical to those used for the serum assay.

c) Validation of the urinary assay procedure

A stock solution of (*R*,*S*)-ibuprofen (1 mg/10 ml) was prepared in acetonitrile. Into six separate 10 ml volumetric flasks were pipetted 0.02, 0.05, 0.1, 0.2, 1.0 and 2.0 ml of the stock solution. The solutions were evaporated gently under nitrogen gas. Blank drug free urine was added to the flasks q.s. 10 ml to give final solutions of 0.1, 0.25, 0.5, 1.0, 5 and 10 µg/ml of each enantiomer. Aliquots (0.5 ml) of these standards were transferred into different tubes and stored at -20^{0} C. On each day of analysis one set of these tubes (0.1 – 10 µg/ml) were analysed together with the samples. Calibration curves were constructed by plotting peak area ratios (ibuprofen enantiomer:(*S*)-flurbiprofen derivatives) against the concentration of each enantiomer and subjecting this data to linear regression analysis. The concentration of each enantiomer in urine samples were determined by comparing their respective peak area ratios to the calibration curve prepared.

The accuracy and within day variation of the assay was assessed by analysing six spiked serum samples with the following enantiomer concentrations: 0.1, 1.0 and 10 µg/ml. The precision and accuracy of the assay was determined for each enantiomer by calculating the coefficient of variation (standard deviation/mean x 100%) and mean percentage difference (mean concentration - actual concentration /

actual concentration x 100%). The recovery of the extraction procedure was determined by comparing peak areas obtained with direct injections of standard solutions of equivalent concentrations.

The day to day variation of the assay was determined by analysing spiked serum samples of 0.1, 1.0 and 10 µg/ml enantiomer concentrations for six consecutive days. The coefficient of variation and accuracy was calculated as before.

2.3 Results and discussion

In this present study, an indirect method of enantioseparation for ibuprofen was favoured over the use of CSPs for the following reasons:

i) achiral columns, especially reversed phase columns, are more robust and stable compared with CSPs. This is especially important when large numbers of biological samples have to be analysed;

ii) the cost of achiral columns are considerably lower than that of CSPs. This factor is inter-related to i) above;

iii) to date the resolution achieved by indirect separation of ibuprofen enantiomers is much better than those by CSPs. This is obvious when comparing published direct methods (Hermansson and Eriksson, 1986; Geisslinger *et al.*, 1992; Nicoll-Griffith *et al.*, 1988 and Petersson *et al.*, 1991) with indirect methods (Avgerinos and Hutt, 1987; Rudy *et al.*, 1990). Moreover, while CSPs are relatively efficient at separating enantiomers, they are less efficient at enantiomer resolution from achiral or endogenous interferences. Thus for bioanalysis, indirect separations have a distinct advantage.

iv) the CDA used, (*R*)-NEA, is available in high optically purity (>99%), and to date there is no evidence of racemization occurring with the derivatization procedure of Hutt *et al.*, (1986).

v) (*R*)-NEA, is highly fluorescent and increases assay sensitivity.

A wide variety of C_{18} phases were evaluated as to their suitability for use in the assay. It was found that the Resolve C_{18} column gave the best separation between the diastereomeric amides of ibuprofen (α_I = 1.16), flurbiprofen (α_F = 1.29) as well as between (*S*)-flurbiprofen and (*R*)-ibuprofen (α_{FI} = 1.23), as compared to columns like Partisil ODS-3 (α_I = 1.16 ; α_F = 1.28 ; α_{FI} = 1.03), Hypersil C_{18} (α_I = 1.10; α_F = 1.05; α_{FI} = 1.17), Spherisorb ODS-1 (α_I = 1.10 ; α_F = 1.11; α_{FI} = 1.20), Spherisorb ODS-2 (α_I = 1.0 ; α_F = 1.0 ; α_{FI} = 1.21), and Ultracarb C_{18}. (α_I = 1.08 ; α_F = 1.19 ; α_{FI} = 1.19) using the same mobile phase. The other columns were discarded on the basis that they did not give equally good separation between the four diastereomeric peaks.

2.3.1 Synthesis of (*R*)-1-(naphthen-1yl)ethylamides of (*R*)- and (*S*)-ibuprofen

In order to fully characterise and validate an enantiospecific analytical method based on the indirect approach, it is advantageous to have available synthetic samples of the diastereomeric derivatives for use as reference standards The synthesis of the required amide diastereoisomers of ibuprofen was therefore carried out by reaction of the racemic drug with (*R*)-NEA using the mixed anhydride method. The two required derivatives were separated and isolated by preparative TLC, examination of the TLC plate after development showed two distinct bands with R_f values of 0.4 and 0.62 respectively. Comparison of the chromatographic characteristics of the prepared derivatives with derivatized standards of pure enantiomers of ibuprofen, indicated that the faster running band was due to the (*R*)-1-(naphthen-1yl)ethylamide of (*S*)-ibuprofen (*S*- acid:*R*-amine) and that the slower was due to that of the (*R*)-1-(naphthen-1yl)ethylamide of (*R*)-ibuprofen (*R*-acid:*R*-amine). The fluorescence spectra for the diastereomeric amides of ibuprofen are summarised in Appendix 1. The fluorescence spectra of both amides are identical with an excitation and emission maxima of 290 and 330 nm respectively. These values were thus used as detector settings for the HPLC fluorescence detector.

2.3.2 Enantiospecific analysis of ibuprofen in serum

a) Derivatization procedure

The derivatization procedure adopted in this study is based on that by Avgerinos and Hutt (1987). The coupling reaction involves a diimide, 1-(3-dimethyl aminopropyl)-3-ethylcarbodiimide hydrochloride (CDI) and 1- hydroxybenzotriazole (HOBT) acting as both activating and dehydrating agents (Figure 2.1) . In the amounts used, it was found that the yield was essentially quantitative (Avgerinos and Hutt, 1987). In the present study, the derivatization yield was found to be 97.5 and 97.8% for (*R*)- and (*S*)-ibuprofen respectively. This was determined by comparing peak areas obtained for derivatized ibuprofen and comparing with those obtained by direct injection of equivalent amounts of the respective amides synthesised in section 2.2.5(b).

Although the reaction times for derivatization were relatively long, the amides were found to be relatively clean and free from interferences when analysed by HPLC However, excess (*R*)-NEA reagent eluted as a large resolved peak (R_t = 28.2 mins) after the (*S*)-ibuprofen derivative peak. In order to remove the excess amine from the derivatization samples, it was necessary to pass the reaction mixture through a 1 ml SPE silica cartridge which effectively removed the excess reagent without retaining the amides. The cartridges were further eluted with dichloromethane:acetonitrile (9:1, v/v) in an effort to totally elute any retained amides. This SPE clean-up step was relatively simple, efficient and more effective than back extraction with 1.0M HCl. An added advantage was observed when analysing serum and urine samples as it was apparent that the SPE cartridge removed a large portion of co-extracted endogenous material, resulting in a cleaner chromatograms.

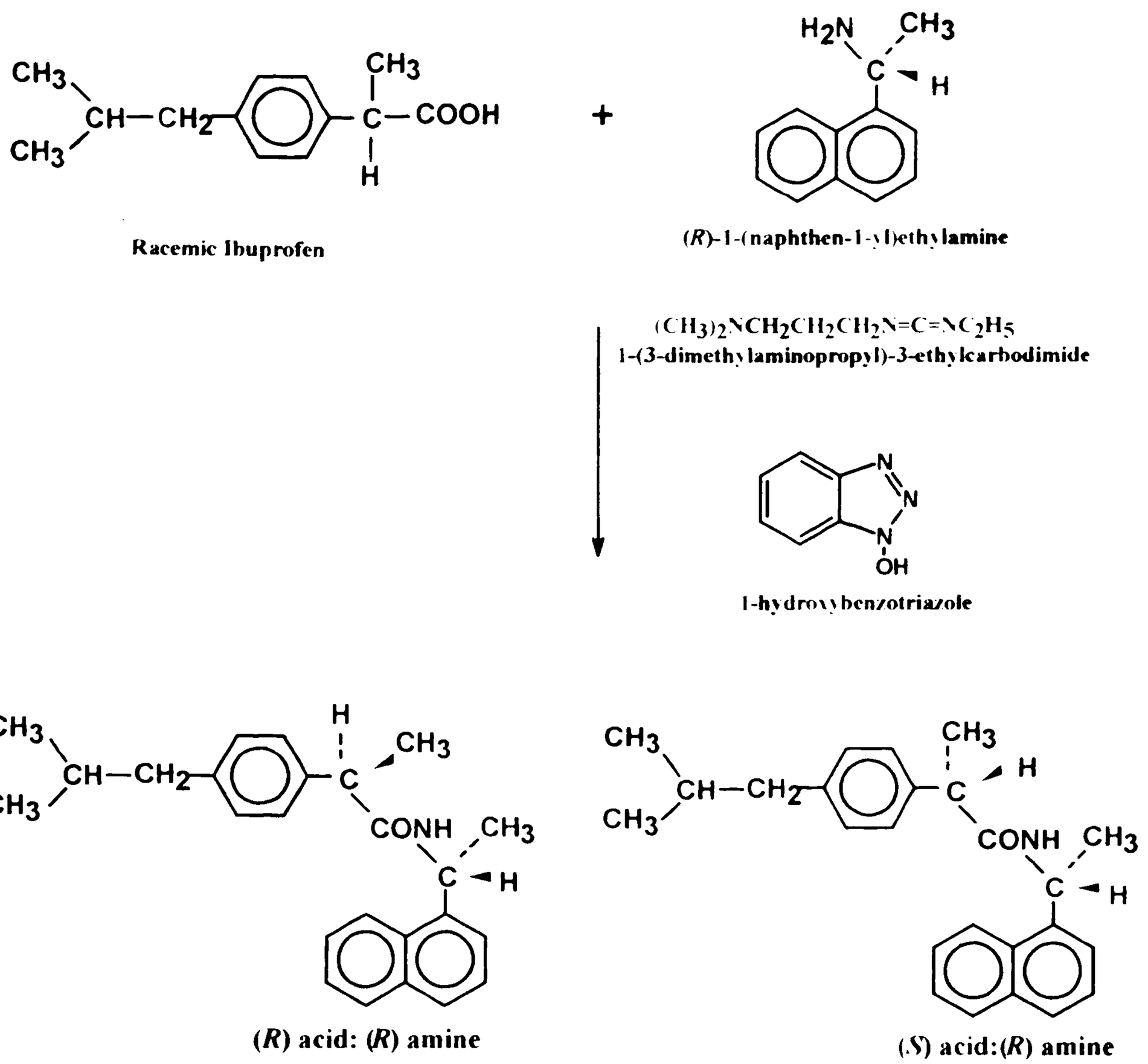


Figure 2.1: Derivatization of the enantiomers of ibuprofen with (*R*)-1-(naphthen-1-yl)ethylamine in the presence of 1-hydroxybenzotriazole and 1-(3-dimethylaminopropyl)-ethylcarbodiimide to yield a pair of diasterosiomeric ibuprofenyl-1-(naphthen-1yl)ethylamides.

b) Extraction procedure

From the chromatograms of authentic and "spiked" serum samples (Figure 2.2) it can be seen that the peaks corresponding to the derivatives of (*R*)- and (*S*)-ibuprofen eluted with retention times of 22.2 and 25.6 minutes and were free from interfering substances. However, for flurbiprofen, the peak corresponding to the

(*R*)-flurbiprofen derivative co-eluted with two other peaks. This interference was only observed in volunteer samples but not in blank or "spiked" serum samples. On further investigation it was found that the interfering peaks were due to the carboxy metabolite of ibuprofen (see Chapter 3). Thus, for quantitative purposes, peak area ratios were calculated using the (*S*)-flurbiprofen derivative peak as internal standard. The values for extraction recoveries from serum are shown in Table 2.4. and are generally greater than 87% for the three concentration levels studied.

d) Validation of assay

The analytical procedure showed good precision and accuracy at all three concentration levels (Table 2.4a) with coefficients of variation less than 8 %. The between day variation also showed equally good consistency (Table 2.4b). Typical calibration curves for both enantiomers are shown in Figure 2.3. Linear regression coefficients are typically better than 0.997. The limit of quantitation was at least 0.1 µg/ml, although this can be lowered considerably by analysing a larger volume of serum or by injecting a larger proportion of the sample on-column. However the level of detection here is sufficient for pharmacokinetic studies involving the total drug enantiomer serum concentrations. The analytical method was developed further for the analysis of unbound serum concentrations (see Chapter 5).

The assay was further validated by analysing a series of samples "spiked" with mixtures of the individual enantiomers at three concentration levels. This validation approach is necessary as biological samples from pharmacokinetic and metabolic studies will probably contain non-racemic mixtures of enantiomers due to stereoselectivity in drug metabolism and disposition (Hutt, 1991). The precision and accuracy values calculated for these analyses are shown in Table 2.5. The measured enantiomeric compositions were in good agreement with the expected values and the variation involved was within acceptable limits at all three "total"

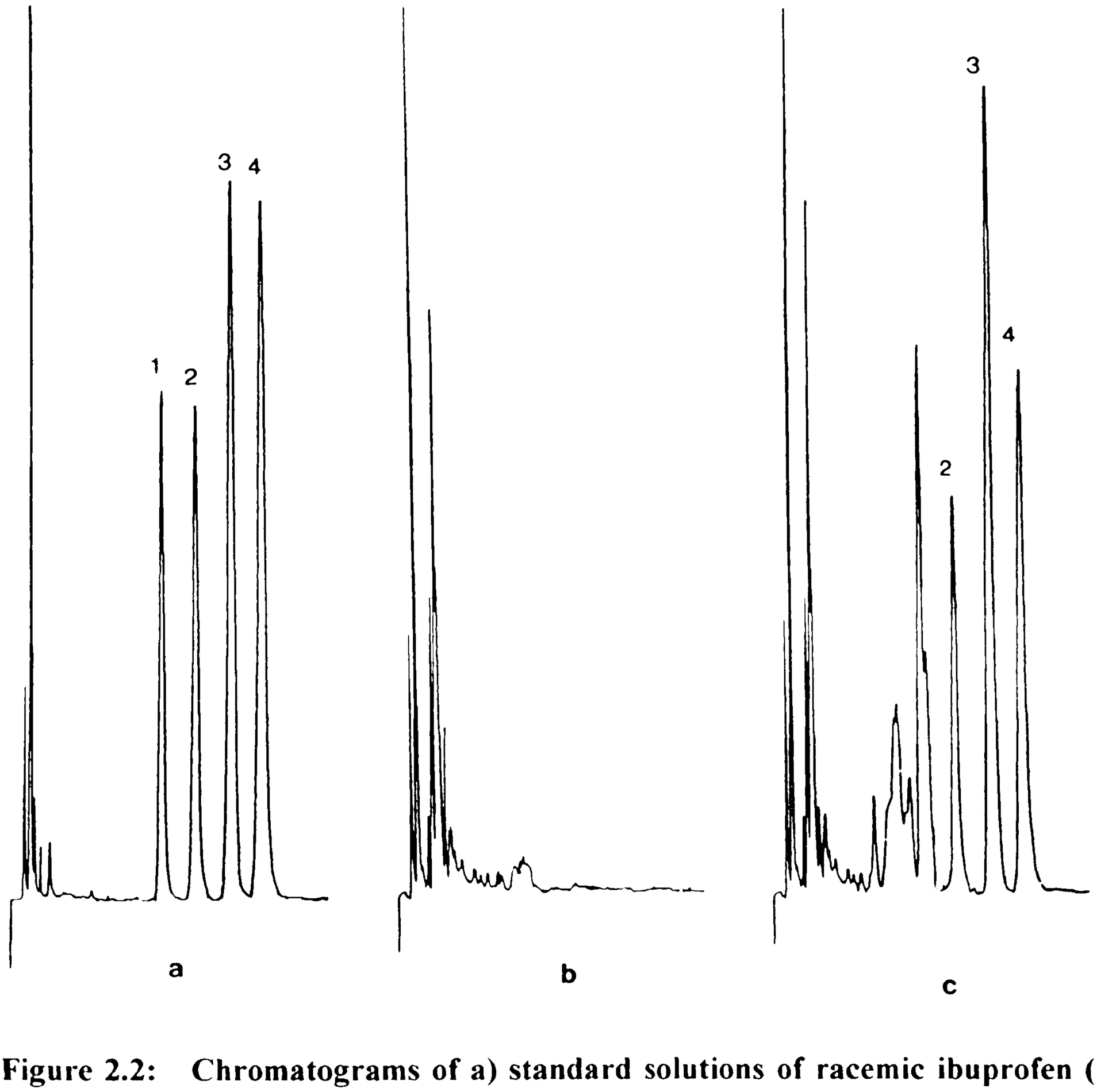


Figure 2.2: Chromatograms of a) standard solutions of racemic ibuprofen (5 µg/ml) derivatized with (*R*)-NEA, b) blank serum sample, c) serum sample of a volunteer 0.75 hr following oral administration of 400 mg of the racemic drug. Retention times peak 1:(*R*)-flurbiprofen, 14.5 min; peak 2: (*S*)-flurbiprofen, 17.8 min (α=1.29, R_s = 1.8); peak 3: (*R*)-ibuprofen, 22.2 min (α=1.23, R_s = 1.6); peak 4: (*S*)-ibuprofen, 25.6 min (α=1.16, R_s =1.3)

Table 2.4: (a) Within day variation, accuracy and extraction recoveries of ibuprofen enantiomers in "spiked" serum samples (n=6) and (b) between day variation and accuracy of ibuprofen enantiomers in "spiked" serum samples (n=6).

(a)

Concentration (µg/ml)	Enantiomer	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference	Recovery (%)
0.1	*R*	0.090 ± 0.005	5.60	-10.0	88.9 ± 5.0
	S	0.091 ± 0.006	6.23	-0.9	88.5 ± 5.5
1.0	*R*	1.04 ± 0.04	3.40	4.0	89.8 ± 3.1
	S	1.09 ± 0.05	4.63	9.0	89.3 ± 4.1
10	*R*	9.93 ± 0.12	1.22	-0.7	87.5 ± 1.1
	S	9.97 ± 0.11	1.12	-0.3	87.5 ± 1.0

b)

Concentration (µg/ml)	Enantiomer	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference
0.1	*R*	0.091 ± 0.008	8.34	-9.0
	S	0.090 ± 0.007	7.80	-10.0
1.0	*R*	0.98 ± 0.06	6.52	-2.0
	S	0.96 ± 0.05	5.65	-4.0
10	*R*	10.01 ± 0.34	3.41	0.1
	S	9.98 ± 0.41	4.08	-0.2

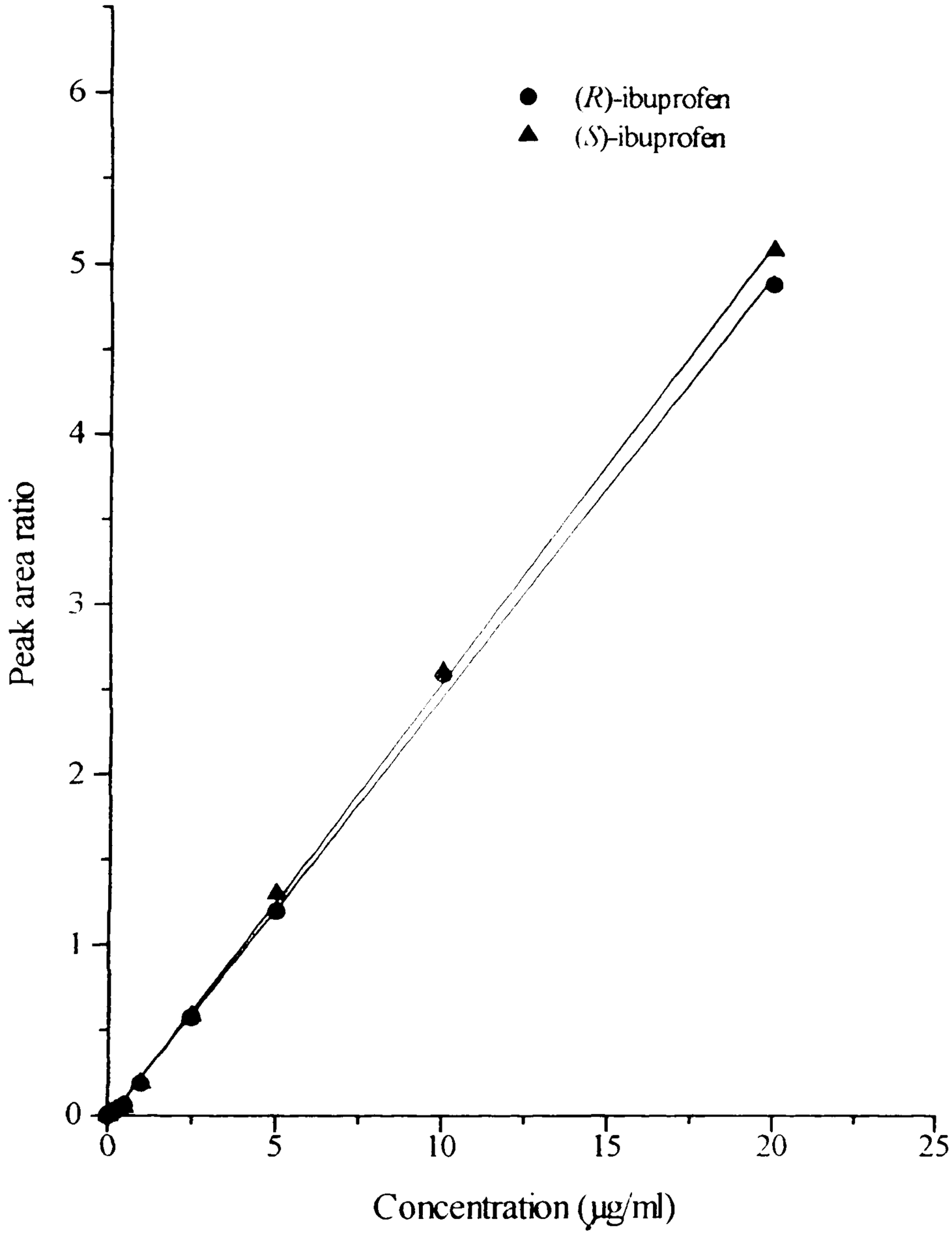


Figure 2.3: Typical calibration curves prepared for the quantitation of ibuprofen enantiomers as their (*R*)-1-(naphthen-1-yl)ethylamides following extraction of the drug from serum.

Table 2.5: Precision and accuracy data for the determination of a series of different enantiomeric compositions of ibuprofen in serum at three different "total" concentrations (n = 6).

a) Low concentration (0.5 μg/ml)

Enantiomer	Theoretical Composition (μg/ml)	Measured Composition (μg/ml)	Mean Percent Difference	Coefficient of Variation (%)
R	0.1	0.102	2.0	4.9
S	0.4	0.387	-1.3	4.1
R	0.2	0.195	-2.5	5.9
S	0.3	0.303	1.0	8.4
R	0.3	0.301	3.3	5.8
S	0.2	0.196	-2.0	6.2
R	0.4	0.405	1.3	4.9
S	0.1	0.090	10.0	6.8

Table 2.5 (cont'd)

b) Medium concentration (2.0 μg/ml)

Enantiomer	Theoretical Composition (μg/ml)	Measured Composition (μg/ml)	Mean Percent Difference	Coefficient of Variation (%)
R	0.4	0.42	5.8	2.23
S	1.6	1.55	-3.1	4.92
R	0.8	0.82	2.1	3.07
S	1.2	1.23	2.3	4.45
R	1.2	1.20	0.1	4.35
S	0.8	0.79	-0.1	4.83
R	1.6	1.58	-1.1	2.89
S	0.4	0.40	0.5	6.80

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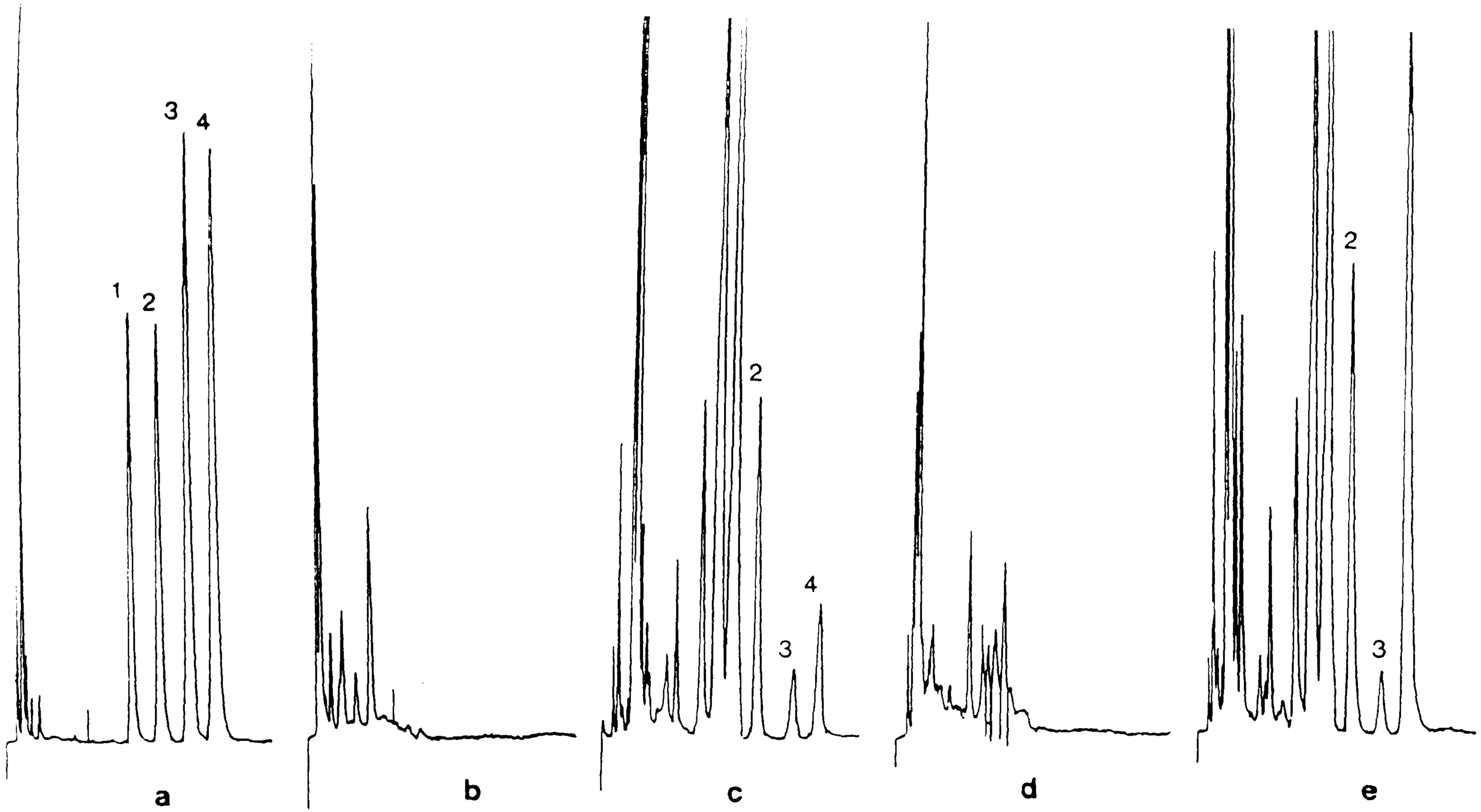


Figure 2.4: Chromatograms of a) standard solutions of racemic ibuprofen (5 µg/ml) derivatized with (*R*)-NEA, b) blank urine sample, c) urine sample of a volunteer, d) alkali treated urine e) alkali treated urine sample of a volunteer 2 - 4 hr following oral administration of 400 mg of the racemic drug. Retention times peak 1:(*R*)-flurbiprofen , 14.5 min; peak 2:(*S*)-flurbiprofen, 17.8 min (α=1.29, R_s = 1.8); peak 3: (*R*)-ibuprofen, 22.2 min (α=1.23, R_s = 1.6); peak 4: (*S*)-ibuprofen, 25.6 min (α=1.16, R_s =1.3);

Table 2.6: (a) Within day variation, accuracy and extraction recoveries of ibuprofen enantiomers in "spiked" urine samples (n=6) and (b) between day variation and accuracy of ibuprofen enantiomers in "spiked" urine samples (n=6).

(a)

Concentration (µg/ml)	Enantiomer	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference	Recovery (%)
0.1	*R*	0.092 ± 0.004	4.31	-8.0	92.7 ± 4.0
	S	0.094 ± 0.004	4.54	-6.0	94.1 ± 4.3
1.0	*R*	0.95 ± 0.03	3.29	-5.0	92.4 ± 3.0
	S	0.96 ± 0.03	3.34	-4.0	93.1 ± 3.1
10	*R*	10.02 ± 0.30	2.96	0.2	95.7 ± 2.8
	S	9.98 ± 0.37	3.66	-0.2	97.1 ± 3.6

b)

Concentration (µg/ml)	Enantiomer	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference
0.1	*R*	0.095 ± 0.006	6.67	-5.0
	S	0.091 ± 0.005	5.96	-9.0
1.0	*R*	0.96 ± 0.04	4.47	-4.0
	S	0.94 ± 0.05	5.01	-6.0
10	*R*	10.08 ± 0.35	3.51	0.8
	S	10.05 ± 0.42	4.21	0.5

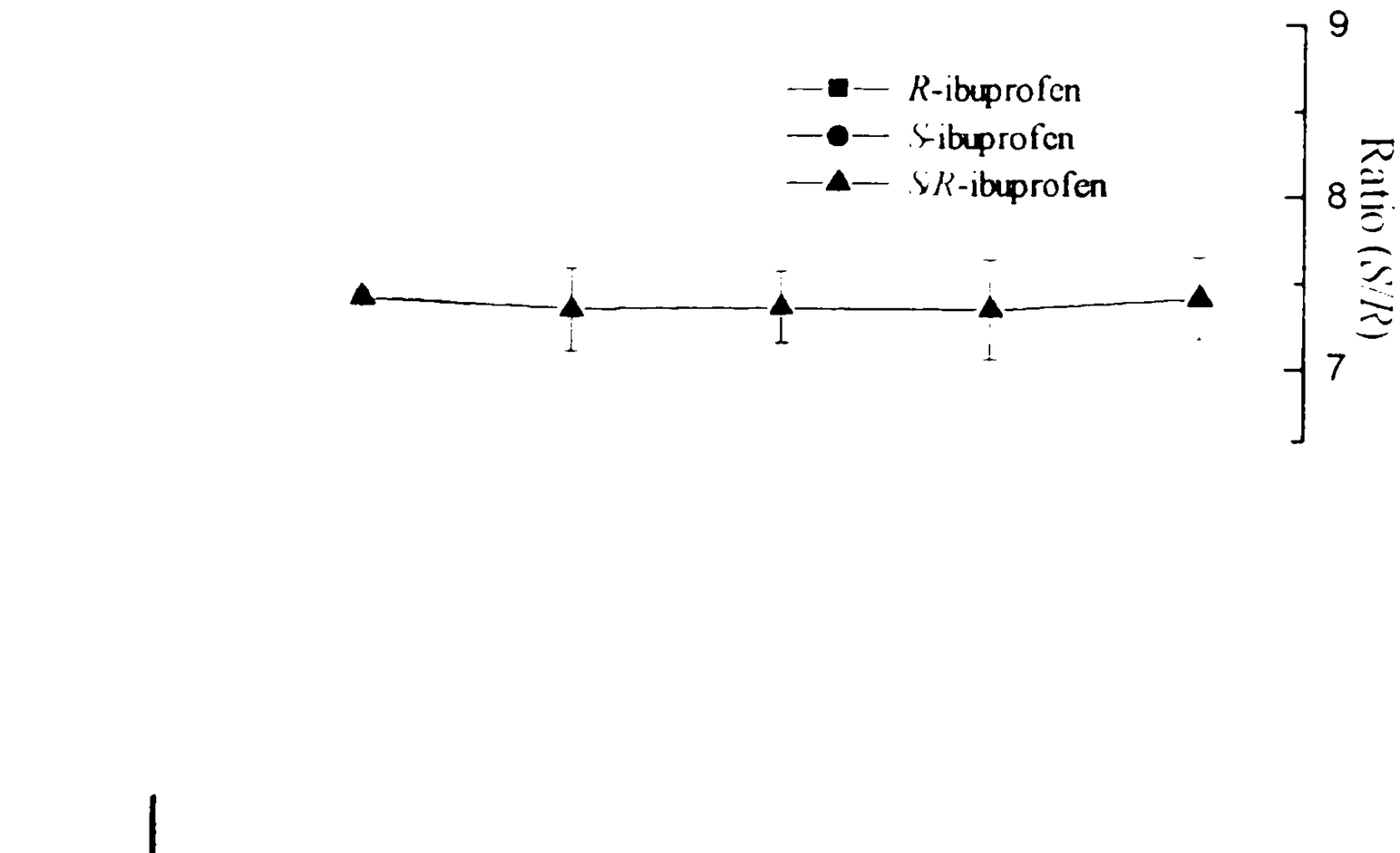


Figure 2.5: Effect of hydrolysis reaction time on the enantiomer concentration and ratios (*S/R*) of ibuprofen in a urine sample of a volunteer (2-4 hr) following the oral administration of 400 mg of the racemic drug (mean ± sd; n = 3).

peaks were observed in the chromatograms of blank and alkali treated urine samples at the retention times of the derivatives of (*R*)- and (*S*)-ibuprofen and (*S*)-flurbiprofen (22.5, 25.6, and 17.8 minutes respectively). However, as with the serum samples, the (*R*)-flurbiprofen derivative co-eluted with the carboxy metabolite peaks and as such (*S*)-flurbiprofen was used as internal standard.

b) Validation of urinary assay

The extraction recoveries for both enantiomers of ibuprofen in urine was approximately 90% while the within day precision and accuracy are generally good and comparable to the serum assay (Table 2.6a). The between day variation experiments also showed good consistencies (Table 2.6b), and daily calibration curves have regression coefficients of better than 0.997 for both enantiomers (Figure 2.6).

2.5 Summary

In summary, a reliable and accurate indirect stereospecific assay for ibuprofen enantiomers in serum and urine was developed and validated. There was no evidence of racemization or non-chiral differentiation in the analytical procedure. The use of fluorescent detection was possible by using (*R*)-NEA as CDA, which enhanced analytical sensitivity and selectivity. The application of the analytical methodology to human pharmacokinetic studies following the oral administration of racemic ibuprofen to healthy volunteers is presented in Chapters 6 and 7.

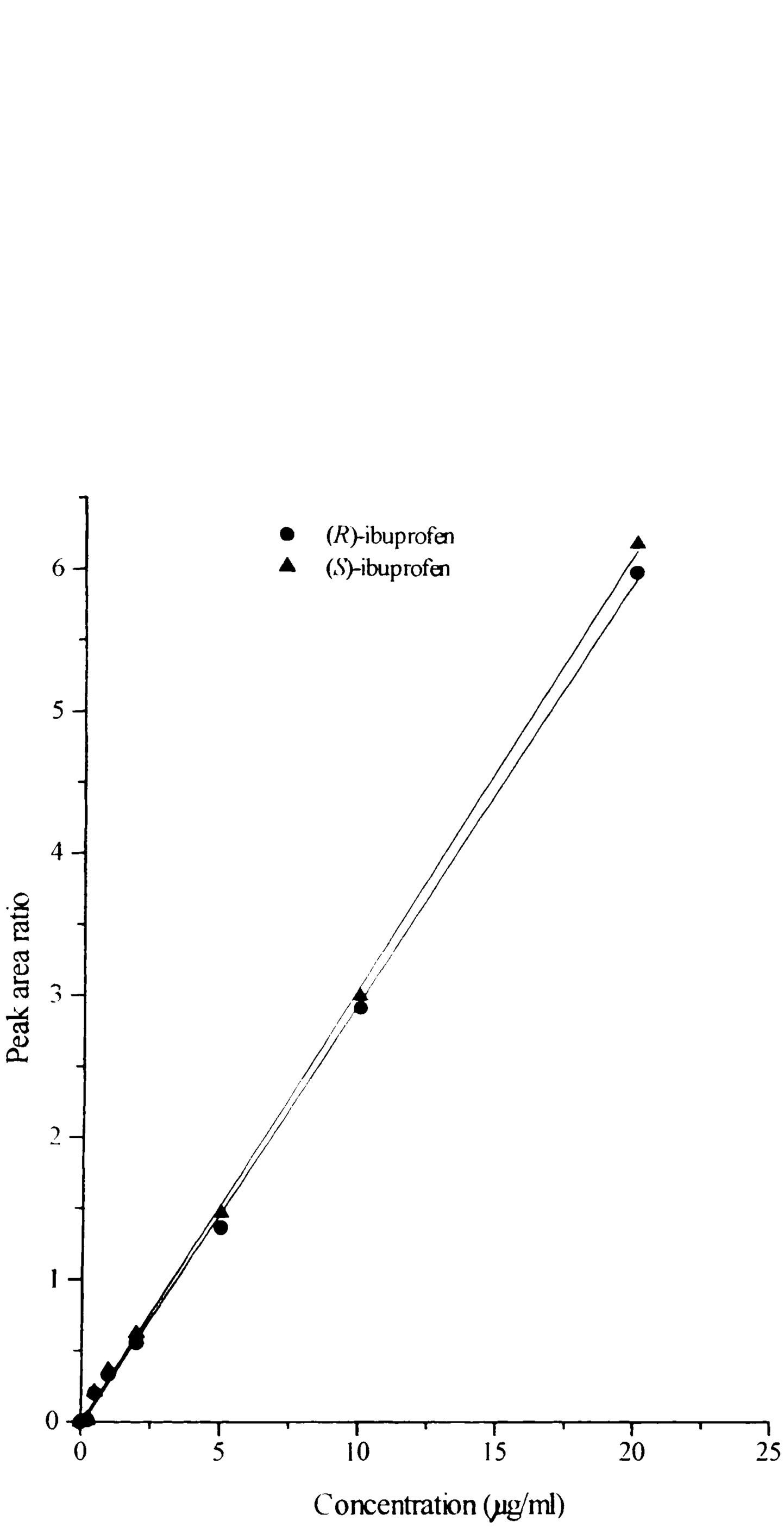


Figure 2.6: Typical calibration curves prepared for the quantitation of ibuprofen enantiomers as their (*R*)-1-(naphthen-1-yl)ethylamides following extraction of the drug from urine.

CHAPTER 3

Chromatographic Resolution, Isolation and Chiroptical Characterisation of Carboxyibuprofen Stereoisomers.

3.1 Introduction

Ibuprofen undergoes metabolic oxidation of its isobutyl side-chain to form hydroxyibuprofen and carboxyibuprofen as major metabolites in both animals and man (Mills *et al.*, 1973). Following the administration of (*R,S*-)-ibuprofen to humans, both enantiomers of hydroxyibuprofen are formed, the *S*-enantiomer being predominant (Baillie *et al.*, 1989; Kaiser *et al.*, 1976). The formation of carboxyibuprofen introduces a second chiral centre into the molecule, with the result that following administration of the racemic drug, four stereoisomers may be found in urine (Kaiser *et al.*, 1976). The determination of the stereochemical composition of the carboxy metabolite in urine following the administration of (*RS*)-ibuprofen has proved to be problematic due to the difficulties associated with chromatographic resolution of the four stereoisomers, as described below, together with the lack of readily available analytical standards.

One of the earliest attempts to resolve the stereoisomers of the two metabolites was published by Kaiser *et al.*, (1976). In this method, urine samples were extracted by successive column chromatography prior to separation using GLC as (*S*)-1-phenylethylamide derivatives. The *R*- and the *S*- enantiomers of the hydroxy metabolite were well resolved but only three peaks were obtained out of a possible four for the carboxy metabolite. Peak assignment for the hydroxy metabolite enantiomers was carried out by analysing urine samples from volunteers following administration of with (*S*)-ibuprofen where only (*S*)-hydroxyibuprofen would be present. On the same basis, the two chromatographic peaks corresponding to the diastereoisomers of carboxyibuprofen with the *S*-absolute configuration in the propionic acid side-chain of the molecule were also identified. However, the absolute configuration at the new chiral centre resulting from oxidation of the isobutyl side chain of the molecule were assigned empirically, on the basis that *S*- enantiomers of ibuprofen and hydroxyibuprofen always elute before their *R* antipodes on the GLC system. Thus, in this method, the elution of the carboxyibuprofen diastereomers were in the order *S,S*-, *R,S*-, *S,R*-, and *R,R*-, and there was co-elution of the *S,R*- and *R,S*- isomers.

Subsequent assays by Young *et al.*, (1986) and Baillie *et al.*, (1989) utilised capillary GC-MS and derivatization with (-)-(*S*)- and (+)-(*R*)-phenethylamine respectively. In the study by Baillie *et al.*, (1989), the assignment of the peaks were made with reference to the order of elution suggested by Kaiser *et al.*, (1976), while in that by Young *et al.*, (1986), the order of peak elution was stated to be the same although there was no mention of how this conclusion was arrived at. In both cases, again both the *R,S*- and *S,R*- isomers could not be resolved.

An HPLC method utilising a Pirkle-type chiral stationary phase (CSP) with a covalently bonded (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine chiral selector was published by Nicoll-Griffith *et al.*, (1988), in which ibuprofen and its two metabolites were derivatized to form their corresponding 4-methoxyanilide derivatives. Although the enantiomers of ibuprofen were fully resolved, the enantiomers of hydroxyibuprofen were only partially resolved, while those of carboxyibuprofen were separated only into two separate peaks and the elution order of the stereoisomers could not be assigned. The most successful attempt to date is that of Rudy *et al.*, (1990), where hydroxy and carboxyibuprofen were successfully separated on a reversed-phase HPLC column as their *(S)*-1-phenylethylamide derivatives. Again, due to the lack of authentic standards for the individual carboxyibuprofen diastereomers, the absolute configuration of the material in the individual chromatographic peaks could not be assigned unequivocally and was made by comparison to the original method of Kaiser *et al.*, (1976).

From the publications cited above, it is clear that there are no methods that assign the stereochemistry of the carboxy metabolite to the chromatographic elution order using specific procedures. As the stereochemical composition of this metabolite is important for the examination of the stereoselectivity of ibuprofen disposition, it was considered essential to investigate methods for the chromatographic resolution of the four stereoisomers and investigate possible methodologies for assigning their absolute configuration.

3.2 Experimental

Note on the nomenclature of the stereoisomers of carboxyibuprofen

Chemically, carboxyibuprofen may be designated as 2-[4-(carboxypropyl)phenyl]propionic acid which results in both chiral centres receiving the numerical designation 2. In order to avoid ambiguities in this thesis, the chiral centre in the 2-carboxypropyl side chain, i.e. the chiral centre introduced by metabolism will be referred to as 2', whereas the original chiral centre in the propionic acid moiety will be designated as 2. The trivial name will be used throughout and a particular stereoisomer indicated as, for example, (2'*R*,2*S*)-carboxyibuprofen.

3.2.1 Chemicals and reagents

Hexane, ethanol, methanol, isopropyl alcohol and acetonitrile were obtained from Rathburn (Walkerburn, U.K.). Hydrogen chloride gas, diethyl ether, trifluoroacetic acid (TFA) and all other chemicals (GPR grade) were obtained from BDH (Poole, Dorset, U.K.).

3.2.2 Chromatographic Columns and Supplies

The Partisil silica column (250 x 4.6 mm, 5 µm) and Partisil semi-preparative C_{18} column (100 x 9 mm, 5µm) were obtained from Whatman (Maidstone, Kent, U.K.). Refillable guard columns (10 x 2.1 mm) were packed with pellicular silica (40-63 µm), both obtained from Alltech (Lancs, U.K.). The Spherisorb ODS-1 C_{18} column (150 x 4.6 mm, 5 µm) was obtained from LDC (Stone, Staffs., U.K.). The chiral column was a Chiralpak AD (amylose tris (3,5-dimethylphenylcarbamate)) column (250 x 4.6 mm, 10 µm) used with a matching guard column (5 x 4.6 mm, 10 µm), and was supplied by HPLC Technology Ltd

(Macclesfield, U.K.). The Merck GF_{254} TLC plates were obtained from BDH (Poole, Dorset, U.K.).

3.2.3 Instrumentation

Chiral HPLC was performed using a LDC Constametric 3000 pump, a LDC Spectromonitor 3100 UV detector, a LDC CI 4100 computing integrator (Stone, Staffs) and a Perkin Elmer ISIS 100 autosampler (Beaconsfield, Bucks, U.K.) or a Perkin Elmer Integral 4000 system. Circular Dichroism (CD) measurements were made on a Jasco J600 spectrometer (Halstead, Essex). Proton nuclear magnetic resonance spectra were obtained using a Perkin Elmer R-32 spectrometer (Beaconsfield, Bucks., U.K.) at 90 MHz using tetramethylsilane as internal standard.

3.2.4 Synthesis of carboxyibuprofen diastereomers

A "racemic" mixture of the four diastereoisomers of carboxyibuprofen were synthesised by Dr. J. A. Baker of the Department of Pharmacy, University of Brighton, using the procedure outlined in Appendix 2, and these were used as analytical standards in this study. Mixtures of two diastereoisomers i.e. 2'*S*,2*R*-, 2'*S*,2*S*- and 2'*R*,2*R*-, 2'*R*,2*S*- carboxyibuprofen were synthesised by Dr. K. Afarinkia of the Chemistry Department of King's College London following a scheme of stereoselective reactions, outlined in Appendix 3. The mixtures were purified by semi-preparative reversed-phase HPLC using the Partisil ODS-3 column (5 µm, 100 x 9 mm) with a mobile phase of acetonitrile:/water (25:75, v/v) adjusted to pH 3.0 with HCl. The flow rate used was 2.5 ml/min and detection was by UV, 220 nm. The samples were dissolved in mobile phase to a concentration of 10mg/ml and aliquots (50 µl) were injected onto the HPLC. The eluate containing the peaks of interest were collected from the outlet of the detector and freeze-dried overnight. The purity of the samples before and after semi-preparative chromatography were monitored by reversed-phase HPLC analysis using the Spherisorb ODS-1 column (5 µm, 150 x 4.6 mm) with a mobile phase of acetonitrile:water (75:25, v/v) adjusted to

pH 3.0 with HCl. The flow rate used was 1.0 ml min and detection was by UV, 220 nm.

(*R*)-2-Methyl-3-phenylpropionic acid was also stereoselectively synthesised by Dr. K. Afarinkia using the first stages of the reaction scheme outlined in Appendix 3(b), but with benzyl bromide as starting material, and was used as such.

3.2.5 Synthesis of the dimethyl, diethyl and diisopropyl esters of carboxyibuprofen.

Into a 250 ml ground-glass stoppered flask was transferred 100 ml of methanol. The flask was weighed and HCl gas was bubbled through until an increase in weight of about 3.6 g was recorded. "Racemic" carboxyibuprofen (250 mg) was transferred into a 50 ml round bottom flask and 10 ml of the acidified methanol was added and the mixture refluxed for about two hours. The reaction was monitored by sampling small aliquots of the mixture using a microcapillary tube every 30 minutes. The samples were spotted on a 2.0 cm x 10 cm strip of an aluminium backed silica gel TLC plate, developed using a solvent system of hexane:ethyl acetate (4:1, v/v) and the spots located by short wavelength UV (254 nm). Under these conditions, carboxyibuprofen did not migrate while the corresponding methyl ester was identified by an extra spot with an R_f of 0.4. After the reaction, the contents of the flask was transferred into a 100 ml separating funnel, distilled water (10 ml) was added and the esters were extracted with 3 x 10 ml of diethylether. The ether extracts were combined and dried over Na_2SO_4:$NaHCO_3$ (4:1, w/w), filtered and evaporated to dryness in a rotary evaporator. A colourless oily residue was obtained and the NMR spectra recorded.

The same procedure was repeated using ethanol and isopropanol for the synthesis of the ethyl and isopropyl esters of carboxyibuprofen respectively.

Dimethyl carboxyibuprofen

NMR (90 MHz, $CDCl_3$) δ ppm. 1.13 (d, 3H, $CH_2CHC\underline{H}_3$), 1.45 (d, 3H, $CHC\underline{H}_3$), 2.67 (m, 2H, $C\underline{H}_2CHCH_3$), 2.90 (m, 1H, $CH_2C\underline{H}CH_3$), 3.61 (s, 6H, $COOC\underline{H}_3$), 3.68 (q, 1H, $C\underline{H}CH_3$), 7.17 (dd, 4H, para-substituted phenyl H).

Diethyl carboxyibuprofen

NMR (90 MHz, $CDCl_3$) δ ppm. 1.17 (d, dt, 9H, $C\underline{H}_3CHCH_2$; $C\underline{H}_3CH_2O$), 1.4 (d, 3H, $C\underline{H}_3CHCO$), 2.75 (m, 3H, $CH_3C\underline{H}C\underline{H}_2$), 3.65 (q, 1H, $CH_3C\underline{H}CO$), 4.18 (dq, 4H, $C\underline{H}_2CH_3$), 7.17 (dd, 4H, para-substituted phenyl H).

Diisopropyl carboxyibuprofen

NMR (90 MHz, $CDCl_3$) δ ppm. 1.13 (d, 15H, $C\underline{H}_3CHCH_2$; 4d, $(C\underline{H}_3)_2CHO$), 1.44 (d, 3H, $C\underline{H}_3CH$), 2.6 - 3.0 (complex m, 3H, $CH_3C\underline{H}C\underline{H}_2$), 3.62 (q, 1H, $CH_3C\underline{H}CO$), 4.98 (m, 2H, $(CH_3)_2C\underline{H}O$), 7.17 (dd, 4H, para-subsituted phenyl H).

3.2.6 Chromatographic resolution of carboxyibuprofen diastereoisomers as the dimethyl, diethyl and diisopropyl esters.

Chromatography was carried out using a Chiralpak AD column (10 µm, 250 x 4.6 mm) with a mobile phase of hexane:ethanol (99.5:0.5, v/v), at a flow rate of 1 ml/min. Detection was by UV, 220 nm. The esters were dissolved in mobile phase to obtain a concentration of 0.5 mg/ml and 40 µl aliquots were injected into the HPLC.

3.2.7 Chromatographic resolution of carboxyibuprofen diastereoisomers after derivatization as their (*R*)-1-(naphthen-1-yl)ethylamides.

The derivatization procedure is similar to that described for ibuprofen in section 2.2.5(b). A standard solution of the "racemic" carboxyibuprofen was

prepared in dichoromethane (1 mg/10 ml). To a 10 µl aliquot of the standard solution was added 100 µg each of CDI, HOBT and (*R*)-NEA (100 µl each of a 1 mg/ml solution in dichloromethane). The tubes were tightly capped and the derivatization procedure carried out as before. Reversed phase chromatography was carried out as described in section 2.2.5(c).

The stereochemical purity of the synthesised (*R*)-2-methyl-3-phenylpropionic acid was also determined by converting it into the corresponding amide and subjecting the derivatives to chromatographic analysis as described above.

3.2.8 Chromatographic resolution of carboxyibuprofen diastereoisomers as their free acids.

Chromatography was carried out using a Chiralpak AD column (10 µm, 250 x 4.6 mm). The mobile phase used was hexane:ethanol (92:8, v/v) containing trifluoroacetic acid (0.05% v/v) at a flow rate of 1.0 ml/min. Detection was by UV at 220 nm. A standard solution of carboxyibuprofen (10mg/ml) was prepared in mobile phase and aliquots (20 µl) injected into the HPLC. The (2'*S*,2*R*)-,(2'*R*,2*R*)- and (2'*R*,2*S*)-, (2'*S*,2*S*)- pairs of diastereoisomers that were synthesised and purified as described in section 3.2.4 were also injected and the chromatograms compared.

3.2.9 Chromatographic isolation of the individual carboxyibuprofen diastereoisomers.

Semi-preparative purification of carboxyibuprofen was carried out to isolate the individual diastereoisomers using the same CSP used in section 3.2.8. The mobile phase used was hexane:methanol:ethanol (95:3.5:1.5, v/v) containing 0.05% v/v trifluoroacetic acid. The flow rate used was maintained at 1.0 ml/min and UV detection was set at 220 nm. Carboxyibuprofen standard solutions (25 mg/ml) were prepared in mobile phase and 20 µl aliquots of this solution was injected into the

HPLC. The eluate containing the individual isomers was collected as they eluted from the detector outlet. The eluate was then evaporated gently under nitrogen gas. The stereochemical purity of the isolated carboxyibuprofen isomers was confirmed by re-examination using the CSP.

3.2.10 Circular dichroism spectroscopy of carboxyibuprofen, hydroxyibuprofen and ibuprofen.

Solutions of (*R*)- and (*S*)-ibuprofen, (*R*)- and (*S*)-hydroxyibuprofen, the pure isomers of carboxyibuprofen collected as described in section 3.2.9 and (*R*)-2-methyl-3-phenylpropionic acid were prepared in acetonitrile (1 mg/ml) and examined by circular dichroism. A bandwidth of 1 nm and sensitivity of 50 mdeg was used and the operating temperatures maintained at 25°C. Measurements were made from 185 to 320 nm. For measurements from 185 to 260 nm, a concentration of 0.3 mg/ml and a cell of 0.02 cm pathlength were used. For measurements between 230 to 320 nm, 1 mg/ml solutions and a cell of 1 cm pathlength were used. The CD spectra were normalised for concentration and cell pathlength, and baseline subtraction was carried out for each individual spectrum.

3.2.11 Urinary excretion study following administration of (*S*)-ibuprofen.

(*S*)-Ibuprofen (50 mg) was weighed and filled into an empty size 5 gelatin capsule. The capsule was administered to a healthy young volunteer after an overnight fast and urine excreted over six hours was collected. The urine sample was analysed using the procedures described in section 4.2.5, 4.2.7 and 4.2.8., to determine the chromatographic characteristics of the carboxy metabolites excreted.

3.3 Results and Discussion

Due to the lack of authentic chemical standards of the individual isomers of carboxyibuprofen, these had to be isolated chromatographically. These standards are necessary if the stereoselectivity of the metabolic oxidation processes are to be elucidated. The carboxyibuprofen used as chromatographic standards in the development of separation methods and in the pharmacokinetic studies (Chapters 6 and 7) were synthesised as described in Appendix 2. The syntheses of the pairs of diastereoisomers 2'*R*,2*R*-, 2'*R*,2*S*- and 2'*S*,2*R*-, 2'*S*,2*S*- were used to assist in the stereochemical assignment of the isomers.

3.3.1 Chromatographic resolution of carboxyibuprofen diastereoisomers as their (*R*)-1-(naphthen-1-yl)ethylamides.

A chromatogram of the indirect separation of the carboxyibuprofen isomers after derivatization with (*R*)-1-(naphthen-1-yl)ethylamine, using similar derivatisation and chromatographic conditions as with ibuprofen (Chapter 2) is shown in Figure 3.1(a). Due to the poor resolution obtained for peaks 2 and 3, an observation consistent with previous data (see introduction and references therein), this approach was not pursued further as complete separation would involve impractically long retention times and would therefore be unsuitable for routine analysis. It was therefore decided to examine possible chiral-phase separation of the four stereoisomers. The elution order of the four (*R*)-1-(naphthen-1-yl)ethylamides of the carboxyibuprofen isomers was determined following subsequent studies (see below).

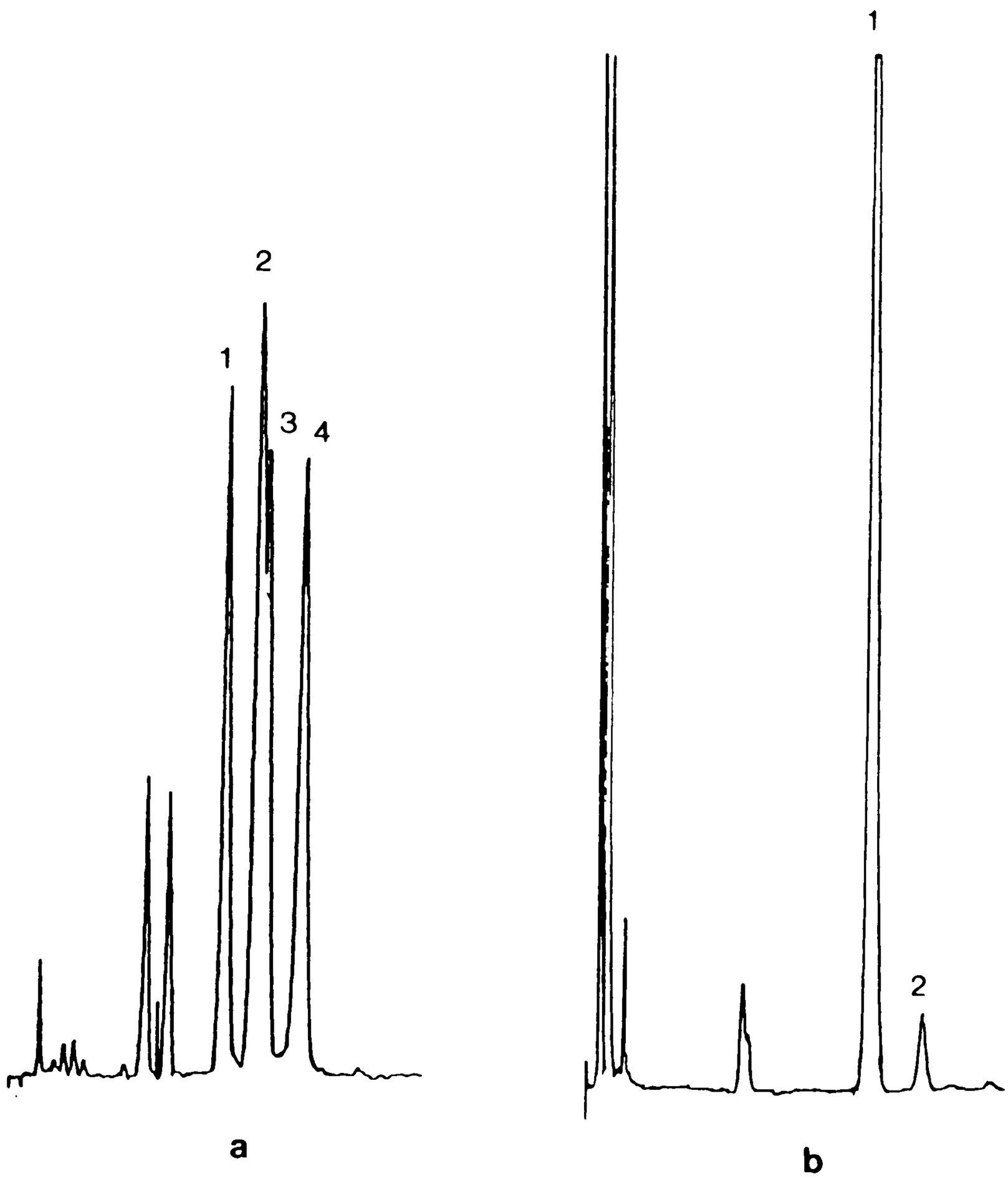


Figure 3.1: Reversed phase chromatographic separation of (a) carboxyibuprofen isomers following derivatization with (*R*)-1-(naphthen-1-yl)ethylamine. Retention times of the diastereoisomers: peak 1: 2'*R*,2*R*- = 13.2 min; peak 2: 2'*S*,2*R*- = 15.1 min (α = 1.25, R_s = 1); peak 3: 2'*R*,2*S*- = 15.6 min (α = 1.04, R_s = 0.17); peak 4: 2'*S*,2*S*- = 17.9 min (α = 1.17, R_s = 0.9) (see text for the determination of the absolute configurations of the individual stereoisomers) (b) (*R*)-2-methyl-3-phenylpropionic acid. Retention time of the *R*- derivative, 17.3 min, retention time of the *S*- derivative, 20.5 min (α = 1.19, R_s = 2.14) [Stationary phase, Resolve C_{18}, 150 x 3.9 mm, 5µm; Mobile phase, acetonitrile:phosphate buffer (0.01M, pH 3.5) (50:50,v/v); Flow rate, 1.5 ml/min; Detection, UV λ = 254 nm].

3.3.2 Synthesis and chromatographic resolution of carboxyibuprofen diastereoisomers as their methyl, ethyl and isopropyl esters.

The successful resolution of enantiomers of carboxylic acids following their conversion into the corresponding neutral esters or amide derivatives using polysaccharide CSPs have been reported in the literature (Shibata *et al.*, 1989). Following this approach, carboxyibuprofen was converted into methyl, ethyl and isopropyl esters following reflux with the appropriate alcohol treated with HCl gas. The structures were confirmed by NMR spectroscopy and their purity determined by TLC analysis using a silica gel GF_{254} a mobile phase of hexane:ethyl acetate (4:1, v/v). These derivatives were analysed using a derivatized amylose CSP (Chiralpak AD). With a solvent system of hexane:ethyl alcohol (99.5:0.5, v/v), the methyl ester derivatives were resolved with baseline resolution (Figure 3.2(a)). However, with the increasing size of the alkyl group, resolution became progressively poorer, accompanied by decreased retention (Figure 3.2 (b and c)). In the case of the isopropyl derivatives, only three peaks were observed. Thus, it is apparent that increasing the hydrophobicity and steric bulk of the ester group led to a decrease in both retention and chiral discrimination.

3.3.3 Chromatographic resolution of carboxyibuprofen as their free acids

Resolution of the carboxyibuprofen isomers in their free acid form was also attempted using the Chiralpak AD column. The mobile phase was modified with the addition of TFA to improve peak symmetry and to reduce retention. The mobile phase composition was adjusted to give adequate separation. With a mobile phase composition of hexane:ethyl alcohol (92:8, v/v) containing 0.05% v/v TFA, baseline separation was achieved at a relatively short run time of 21.5 minutes (Figure 3.3). As these analytical conditions gave the most satisfactory resolution and did not require prior derivatization, this technique was adopted for the subsequent analysis

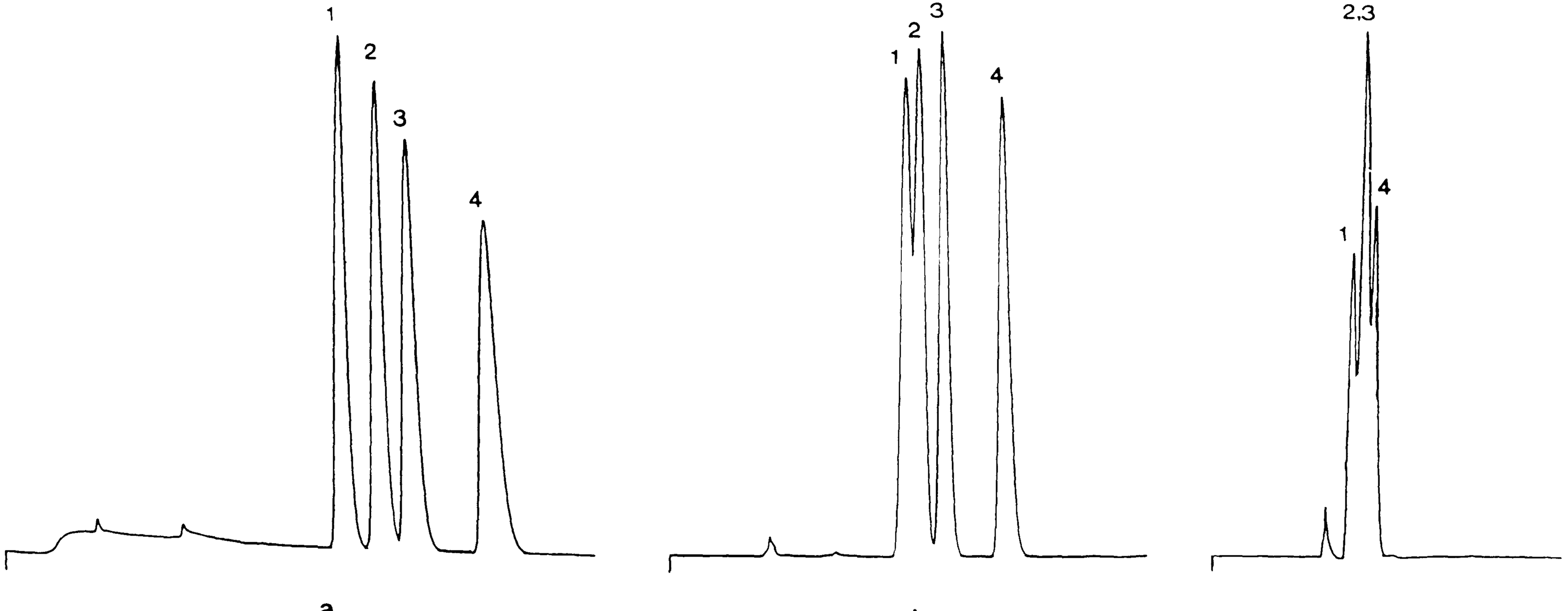


Figure 3.2: Chromatographic resolution of carboxyibuprofen isomers as their respective diesters using a Chiralpak AD CSP;. (a) dimethyl esters, retention times peak 1: 12.2 min; peak 2: 13.5 min(α = 1.16, R_s = 1.1); peak 3: 14.6 min (α = 1.12, R_s = 1.1); peak 4: 17.6 min (α = 1.27, R_s = 1.72); (b) diethyl esters, retention times peak 1: 7.6 min; peak 2: 7.9 min (α = 1.13, R_s = 0.6); peak 3: 9.7 min (α = 1.13, R_s = 0.8); peak 4: 12.1 min (α = 1.32, R_s = 1.9); (c) diisopropyl esters, retention times peak 1: 5.0 min; peak 2: 5.3 min(α = 1.45, R_s = 0.52); peak 3:5.3 min (α = 1.0, R_s = 0) peak 4: 5.6 min (α = 1.20, R_s = 0.46).

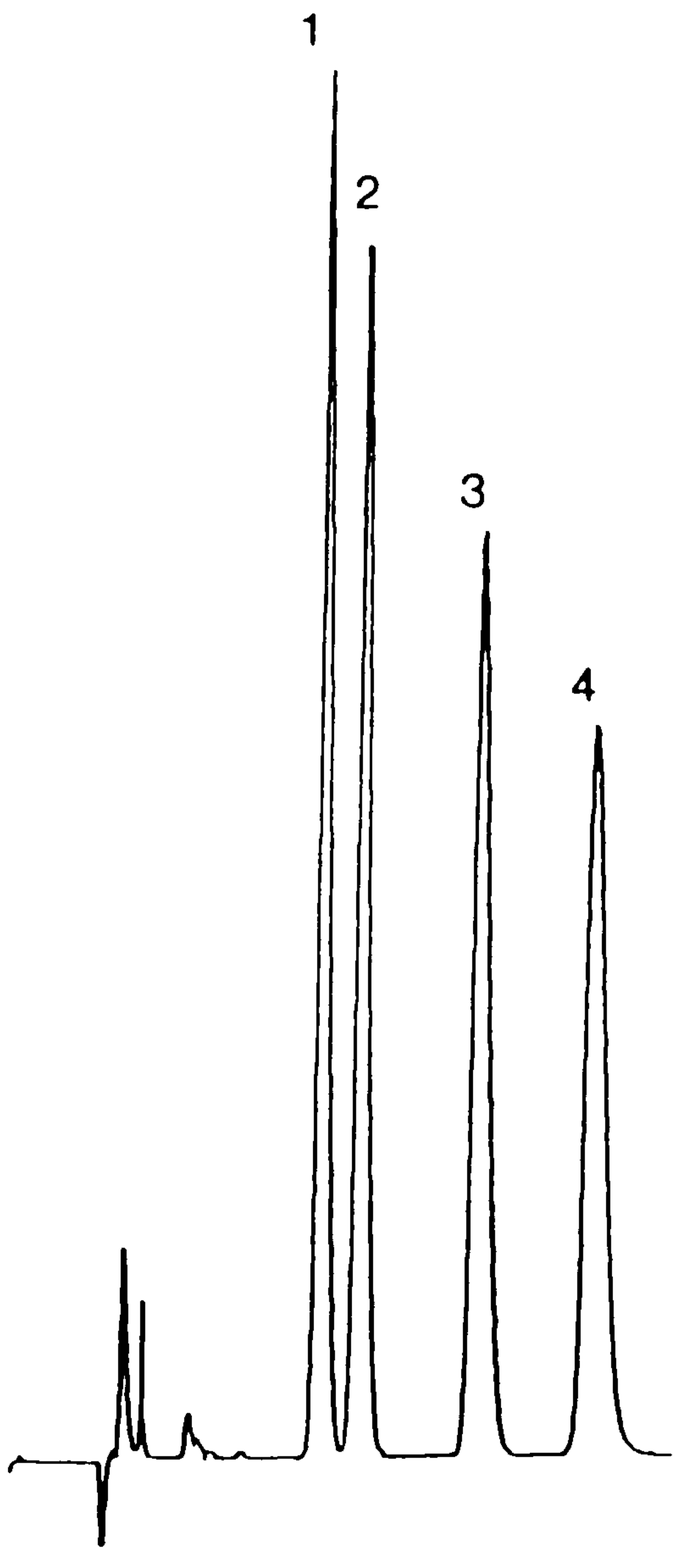


Figure 3.3: Chromatographic resolution of carboxyibuprofen isomers as the free acids using a Chiralpak AD CSP. Retention times peak 1: 11.0 min; peak 2: 12.1 min (α = 1.20, R_s = 1.3); peak 3: 16.9 min (α = 1.5, R_s = 3.3); peak 4: 20.1 min (α = 1.3, R_s = 2.4). For peaks 1 and 3: α = 1.85, R_s = 4.25; peaks 2 and 4: α = 1.84, R_s = 4.55. [Mobile phase, hexane:ethanol (92:8, v/v) with TFA (0.05%, v/v); Flow rate, 1.0 ml/min; Detection, UV λ = 220 nm].

of carboxyibuprofen diastereoisomers in the pharmacokinetic studies (Chapters 6 and 7). For the semi-preparative isolation of the diastereoisomers the mobile phase was modified to hexane:methanol:ethanol (95:3.5:1.5, v/v) with TFA (0.05%, v/v) in order to improve the resolution further, especially between the first two peaks (Figure 3.4). With this improved separation, the amount of substance injected on-column could be increased to 0.5 mg. Following repeated injections (n=30) of the "racemic" mixture of carboxyibuprofen, approximately 3 mg of each stereoisomer were isolated. The stereochemical purity of each isomer was determined by re-injection onto the HPLC. In each case, these were found to be greater than 99% based on peak area measurements (see for example Figure 3.4(b)). The materials isolated were examined by CD spectroscopy (see below).

Attempts to determine the stereochemical purity of the synthesised (*R*)-2-methyl-3-phenylpropionic acid using the CSP were unsuccessful, only one chromatographic peak being obtained. The stereochemical purity was subsequently determined by converting the acid into its corresponding (*R*)-1-(naphthen-1-yl)ethylamide and analysing it by reversed phase HPLC as described in section 3.2.7. The stereochemical purity was determined to be 91% based on peak areas (see Figure 3.1 (b)).

3.3.4 Human urinary excretion study following administration of (*S*)-ibuprofen.

Following the chromatographic resolution and isolation of the four stereoisomers of carboxyibuprofen it was necessary to determine their absolute configuration. As the chiral inversion of (*R*)- to (*S*)-ibuprofen is unidirectional in man, administration of (*S*)-ibuprofen would result in only the urinary excretion of (*S*)-ibuprofen and metabolites having the *S*-configuration in the propionic acid moiety. Therefore, analysis of urine samples after administration of (*S*)-ibuprofen would yield valuable information as to the absolute configuration of the metabolites at the original chiral centre. On analysis of the 0 - 6 hour pooled urine sample from

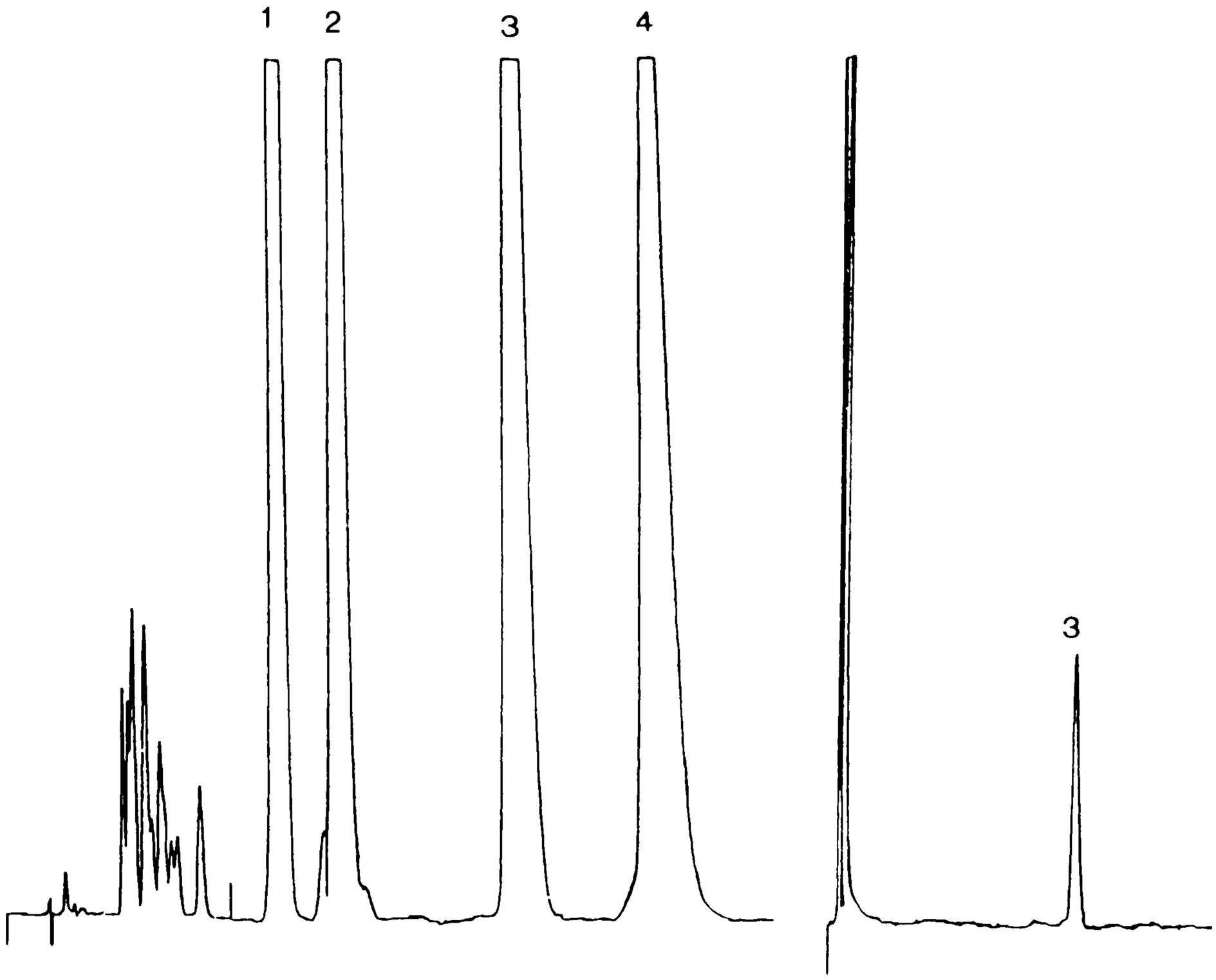


Figure 3.4: (a) Semi-preparative resolution of carboxyibuprofen diastereoisomers using the Chiralpak AD CSP. Retention times, peak 1: 21.2 min; peak 2: 26.2 min (α = 1.23, R_s = 2.9) ; peak 3: 39.4 min (α = 1.57, R_s = 7.6); peak 4: 50.20 min (α = 1.3, R_s = 4.7).[Mobile phase, hexane:methanol;ethanol (95;3.2:1.5, v/v) with TFA (0.05%, v/v); Flow rate, 1.0 ml/min; Detection, UV λ = 220 nm] (b) Chromatographic analysis of peak 3 after semi-preparative isolation. Retention time, 16.9 min (using the system presented in Figure 3.3).

the urinary excretion study for carboxyibuprofen diastereoisomers, only peaks with retention times corresponding to peaks 3 and 4 were obtained (Figure 3.6 (c)). Therefore, peaks 3 and 4 has the *S* absolute configuration at the original chiral centre and by logical deduction the other two peaks, i.e. peaks 1 and 2 have the *R*-absolute configuration at the same centre.

Knowing the absolute configuration at the chiral centre in the propionic acid moiety the CD spectra of the four stereoisomers were examined (see below) in the hope that the configuration of the second chiral centre in each isomer could be deduced. However this proved to be problematical and an alternative approach based on stereoselective synthesis was adopted.

3.3.5 Analysis of 2'*R*,2*R*-, 2'*R*,2*S*- and 2'*S*,2*R*-, 2'*S*,2*S*- pairs of carboxyibuprofen diastereoisomers

The synthesis of the diastereoisomeric pairs of carboxyibuprofen i.e. 2'*R*,2*R*- , 2'*R*,2*R*- pair and 2'*S*,2*R*-, 2'*S*,2*S*- pair was carried out by Dr. K. Afarinkia (Department of Chemistry, King's College London; see Appendix 3). The approach involved stereoselective synthesis of the 2-methylpropionic acid moiety followed by the non-stereoselective introduction of the propionic acid moiety. Also during the course of the synthesis programme, (*R*)-2-methyl-3-phenylpropionic acid was stereoselectively prepared (Appendix 3). Once the synthetic materials were obtained, they were examined by reversed phase chromatography and typical chromatograms are presented in Figure 3.5. After semi-preparative purification on a reversed-phase HPLC system, the samples were then analysed without derivatization using the Chiralpak AD column and the conditions described in section 3.2.8. With the sample containing the 2'*S*,2*R*- and 2'*S*,2*S*- pair of diastereoisomers, two major peaks were seen, with retention times of 11.0 and 20.1 minutes respectively, corresponding to peaks 1 and 4 of the standard "racemic" mixture (Figure 3.6 (a)). Therefore, peaks 1 and 4 both have the *S*-configuration at the newly formed chiral

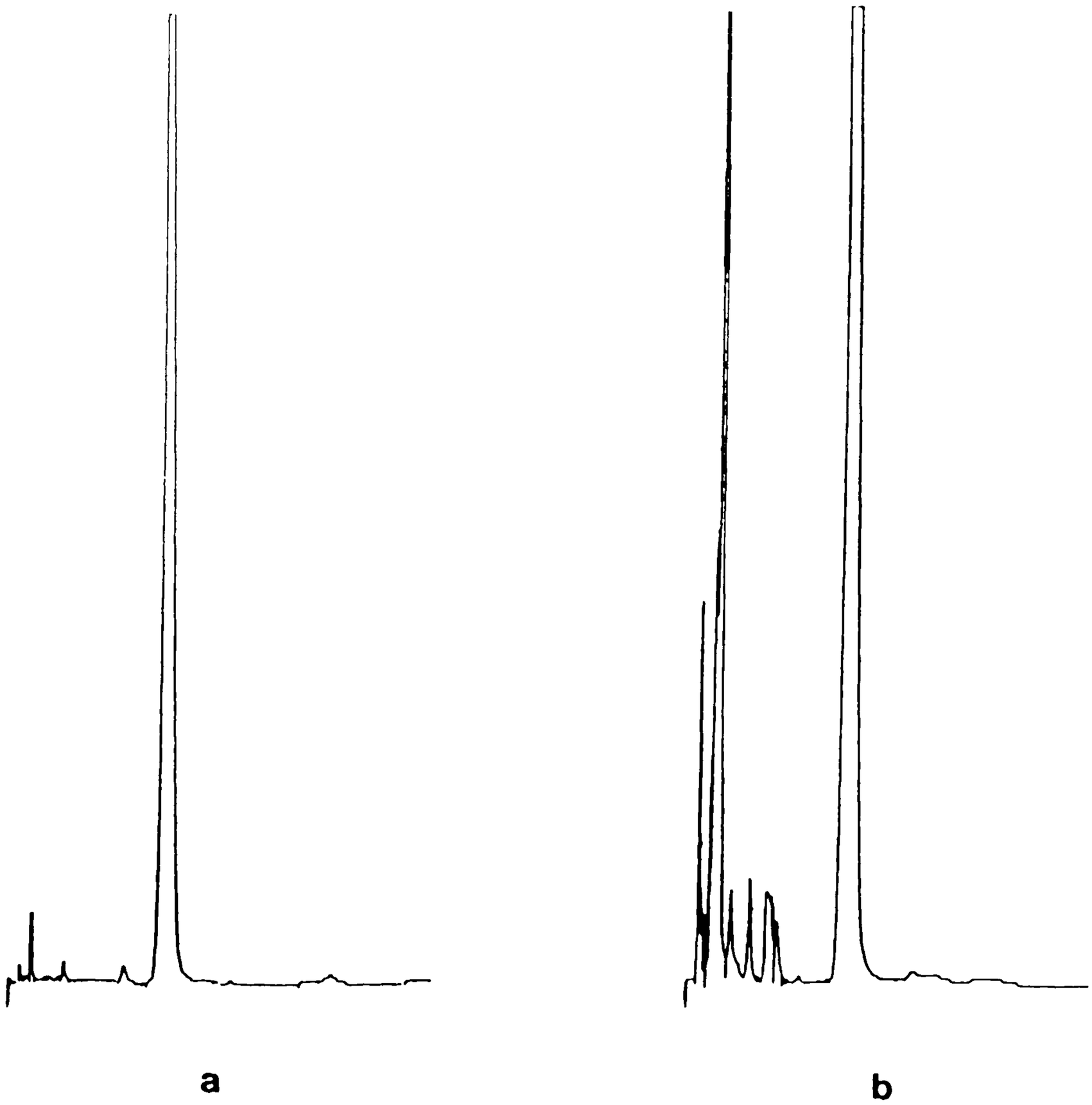


Figure 3.5: Reversed phase analysis of the synthetic diastereoisomeric mixtures of carboxyibuprofen (a) 2'*S*,2*R*-/2'*S*,2*S*- and (b) 2'*R*,2*R*-/2'*R*,2*S*-diastereoisomers, retention time 11.2 minutes. [Stationary phase, Resolve C_{18}, 150 x 3.9 mm, 5 μm; Mobile phase, acetonitrile:water (40:60, v/v;); Flow rate, 1.5 ml/min; Detection, UV λ = 220 nm).

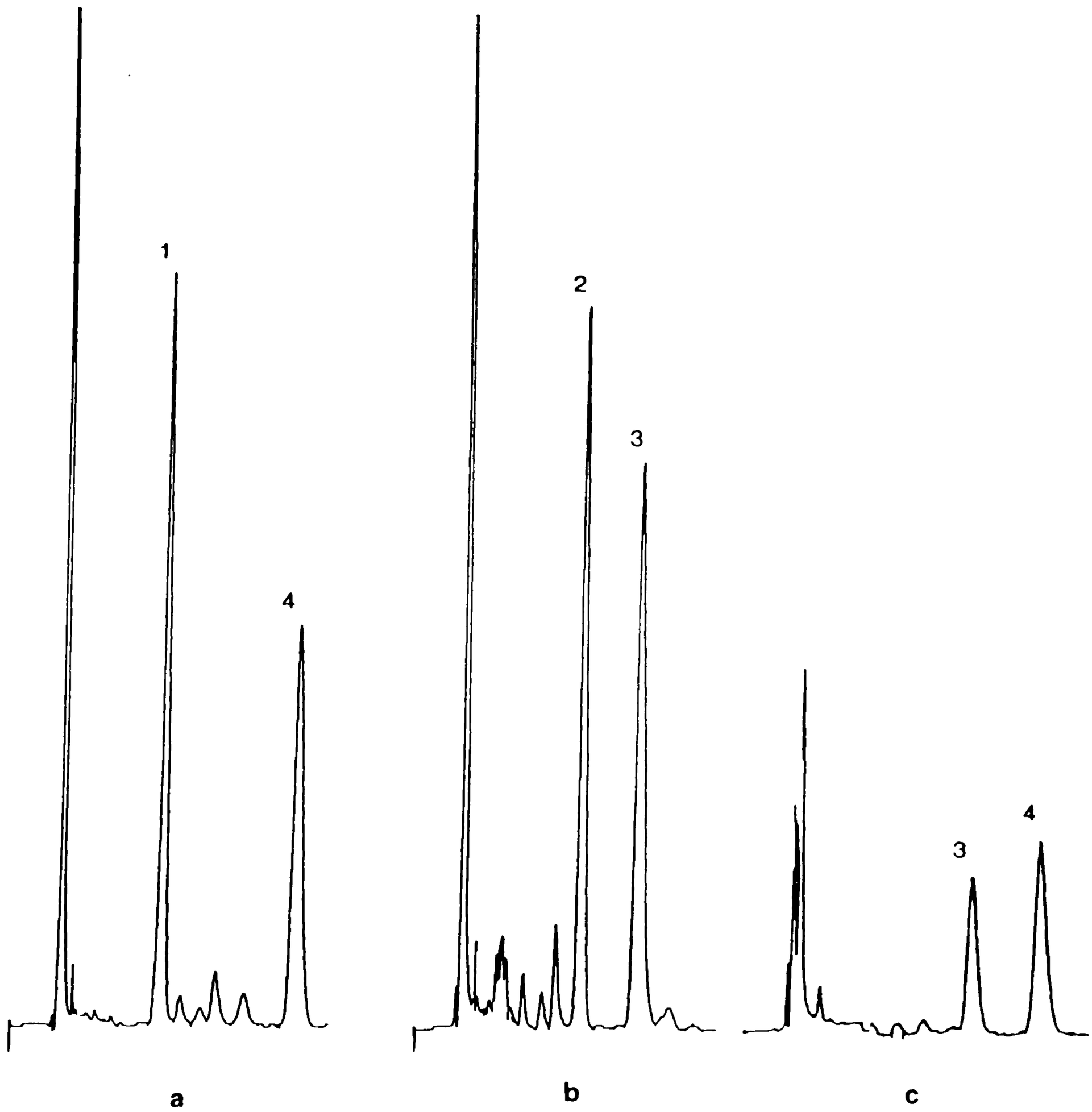


Figure 3.6: Resolution of the synthetic (a) 2'*S*,2*R*- ,2'*S*,2*S*- and (b) 2'*R*,2*R*-, 2'*R*,2*S*- carboxyibuprofen diastereoisomers using the Chiralpak AD after semi-preparative purification. Retention times, peak 1: 11.0 min; peak 2: 12.1 min; peak 3: 16.2 min; peak 4: 20.1 min.: (c) chromatogram of carboxyibuprofen following isolation from the urine of a healthy volunteer after the oral administration of (*S*)-ibuprofen (50 mg). Retention times, peak 3, 16.3 min; peak 4, 20.1 min. (Mobile phase conditions as in Figure 3.3).

centre in the carboxypropyl moiety. With the 2'*R*,2*S*- and 2'*R*,2*R*- pair, two major peaks with retention times of 12.1 and 16.9 minutes were obtained, corresponding to peaks 2 and 3 of the standard "racemic" mixture (Figure 3.6(b)). Therefore, peaks 2 and 3 have the *R*- absolute configuration in the carboxypropyl side chain. Thus, analysis of these stereoselectively synthesised pairs of diastereoisomers gave valuable information on the configuration of the diastereoisomers at the newly formed chiral centre in the carboxypropyl moiety. These data, together with those obtained in the metabolic study are summarised in Table 3.1 and reveals the stereochemical configuration of the isomers with respect to the order of elution from the CSP.

Table 3.1: Stereochemical assignment and chromatographic elution order of the four stereoisomers of carboxyibuprofen.

Peak No:	Rt (min)	Stereochemical Configuration	
		2-carboxypropyl moiety	propionic acid moiety
1	11.0	*S*	*R*
2	12.1	*R*	*R*
3	16.9	*R*	*S*
4	20.1	*S*	*S*

Thus, the chromatographic peaks 1 - 4 have the absolute configurations of 2'*S*,2*R*-, 2'*R*,2*R*-, 2'*R*,2*S*-, and 2'*S*,2*S*- of carboxyibuprofen respectively.

With reference to the order of elution with the indirect separation using reversed phase analysis after derivatization into amides of (*R*)-1-(naphthen-1-yl)ethylamine, the order of elution of the derivatives were 2'*R*,2*R*-, 2'*S*,2*R*-, 2'*R*,2*S*-, and 2'*S*,2*S*- respectively.

3.3.6 Chiroptical properties of carboxyibuprofen diastereoisomers

The concentrations and pathlengths used to determine the CD spectra of the ibuprofen enantiomers were chosen such that the absorbance of the solutions did not exceed 1.5 a.u.f.s. and that the HT voltage of the photomultiplier was less than 700V to ensure that valid CD data were obtained.

The CD spectra of (*S*)-ibuprofen showed bands in three distinct regions (Figures 3.7 to 3.12). The strongest band was observed at 195 nm with a negative Cotton effect, while a relatively weaker one was observed at 225 nm with a positive Cotton effect. Several weakly positive bands were also observed at the region around 260 nm. A comparison with published literature showed that the bands at 225 and 260 nm (Figure 3.7 (a)) for (*S*)-ibuprofen were consistent with those observed with other (*S*)-α–substituted phenylacetic acid derivatives (Barth *et al.*, 1970). The band at 225 nm has been attributed predominantly to $n \rightarrow \pi^*$ transition of the carbonyl group while that at the 260 nm region is probably due ^1L_b transition of the phenyl moiety. With (*R*)-ibuprofen equal and opposite Cotton effects were observed (Figure 3.7(a)). Also, comparison of the CD spectra of (*R*)-ibuprofen with (*R*)-hydroxyibuprofen and that of (*S*)-ibuprofen with (*S*)-hydroxyibuprofen showed almost identical CD spectra (Figure 3.7(b)). The strong bands observed at 195 nm have not been investigated in-depth in previously published work and are most probably due to transitions of the phenyl ring.

With the carboxyibuprofen diastereoisomers, the main differences in the Cotton effects were seen at the 225 nm region, as would be expected as this region is due to the $n \rightarrow \pi^*$ transition of the carbonyl group (Figures 3.8 to 3.10). With the 2'*R*,2*R*- diastereoisomer, the CD spectra in this region were similar to that for (*R*)-ibuprofen although the absorption for (2'*R*,2*R*-)-carboxyibuprofen was slightly stronger . It is therefore evident that the stereochemical configuration of the second chiral carbon in the carboxypropyl moiety exhibits a similar Cotton effect to the original chiral centre at this wavelength region, although the intensity of the Cotton effect of this second chiral centre would appear to be quantitatively weaker. Thus, in

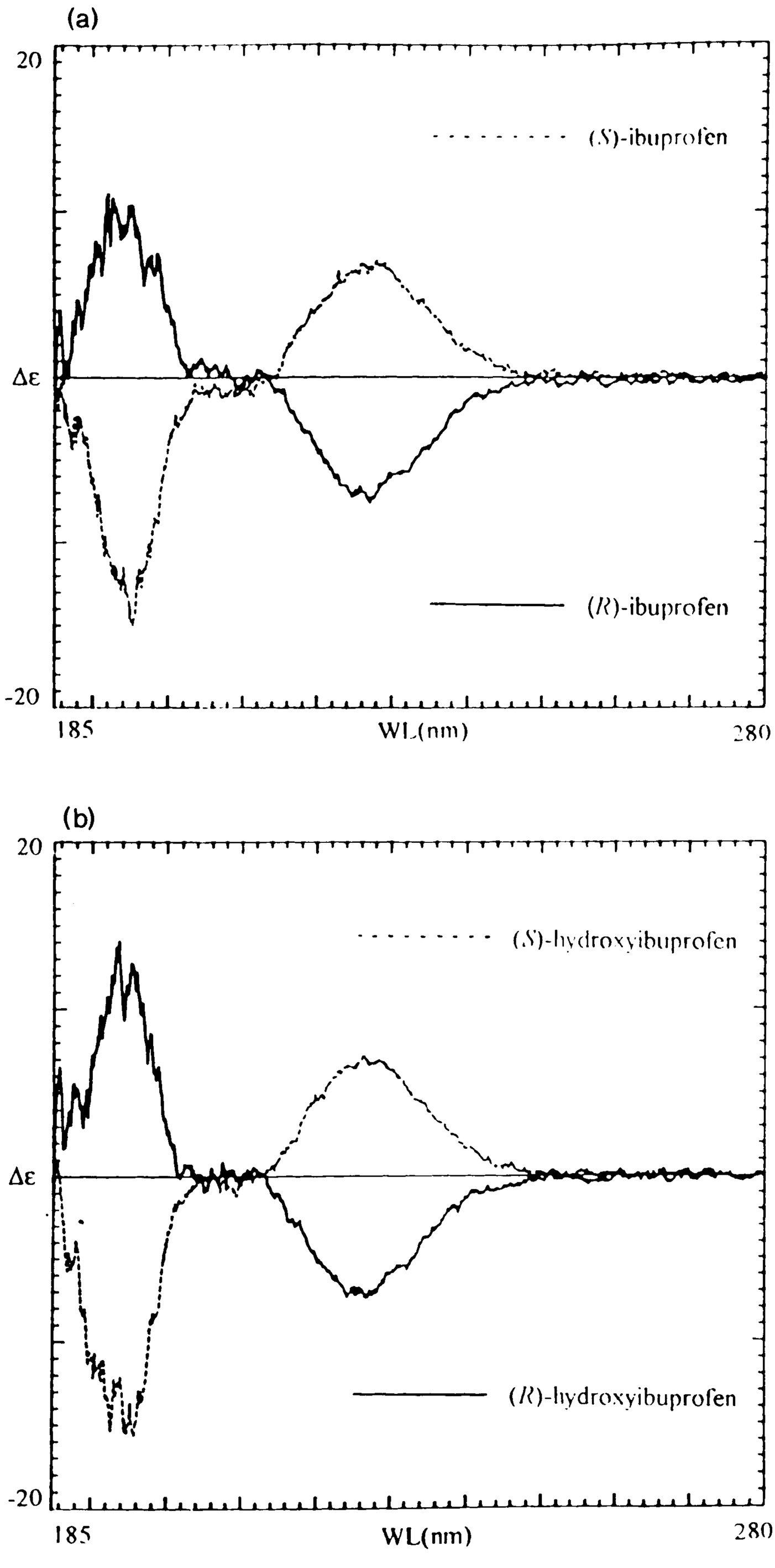


Figure 3.7: CD spectra presented from 185 to 280 nm for (a) (*R*)-ibuprofen and (*S*)-ibuprofen (b) (*R*)-hydroxyibuprofen and (*S*)-hydroxyibuprofen. Samples (0.3 mg/ml) dissolved in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

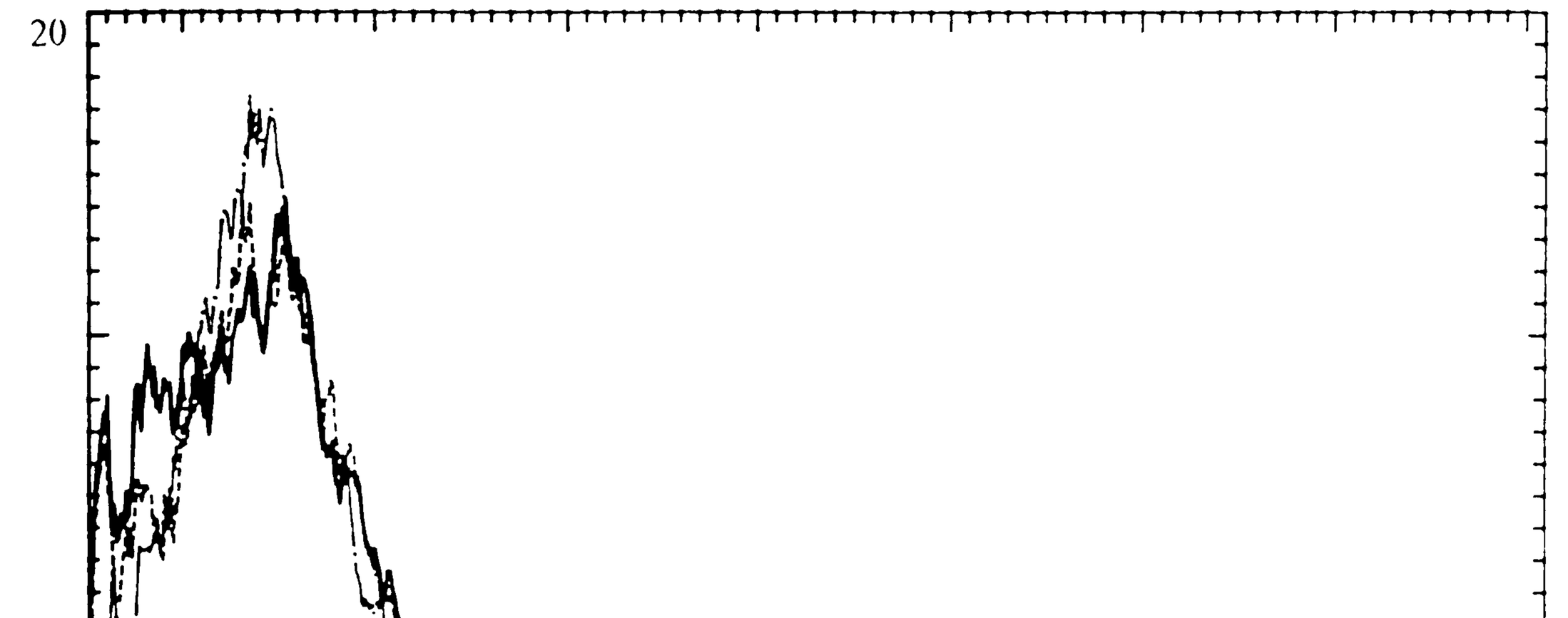


Figure 3.8: CD spectra presented from 185 to 260 nm for (2'*R*,2*R*)-carboxyibuprofen, (2'*S*,2*R*)-carboxyibuprofen and (*R*)-ibuprofen. Samples dissolved (0.3 mg/ml) in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

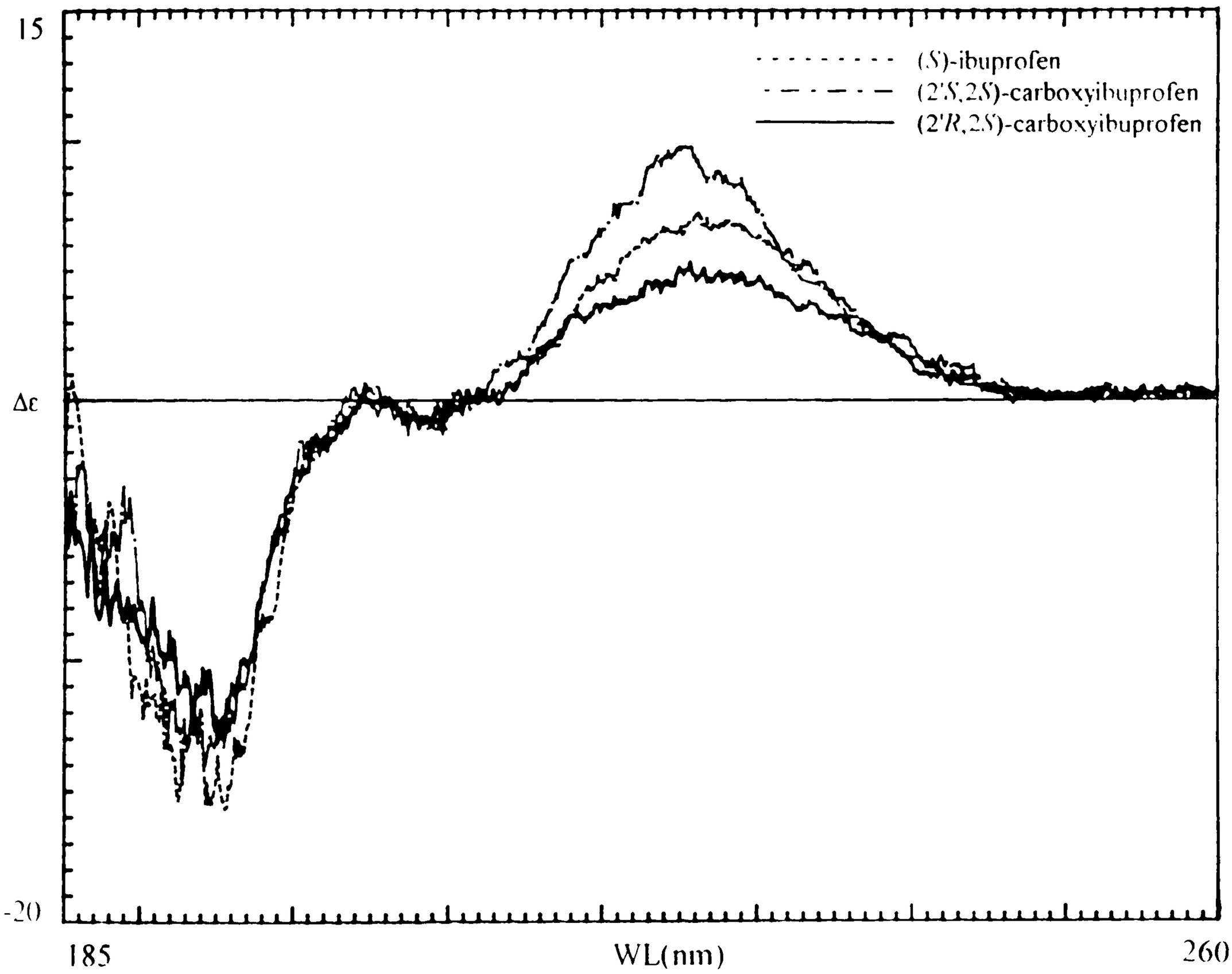


Figure 3.9: CD spectra presented from 185 to 260 nm for (2'*S*,2*S*)-carboxyibuprofen, (2'*R*,2*S*)-carboxyibuprofen and (*S*)-ibuprofen. Samples dissolved in (0.3 mg/ml) in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

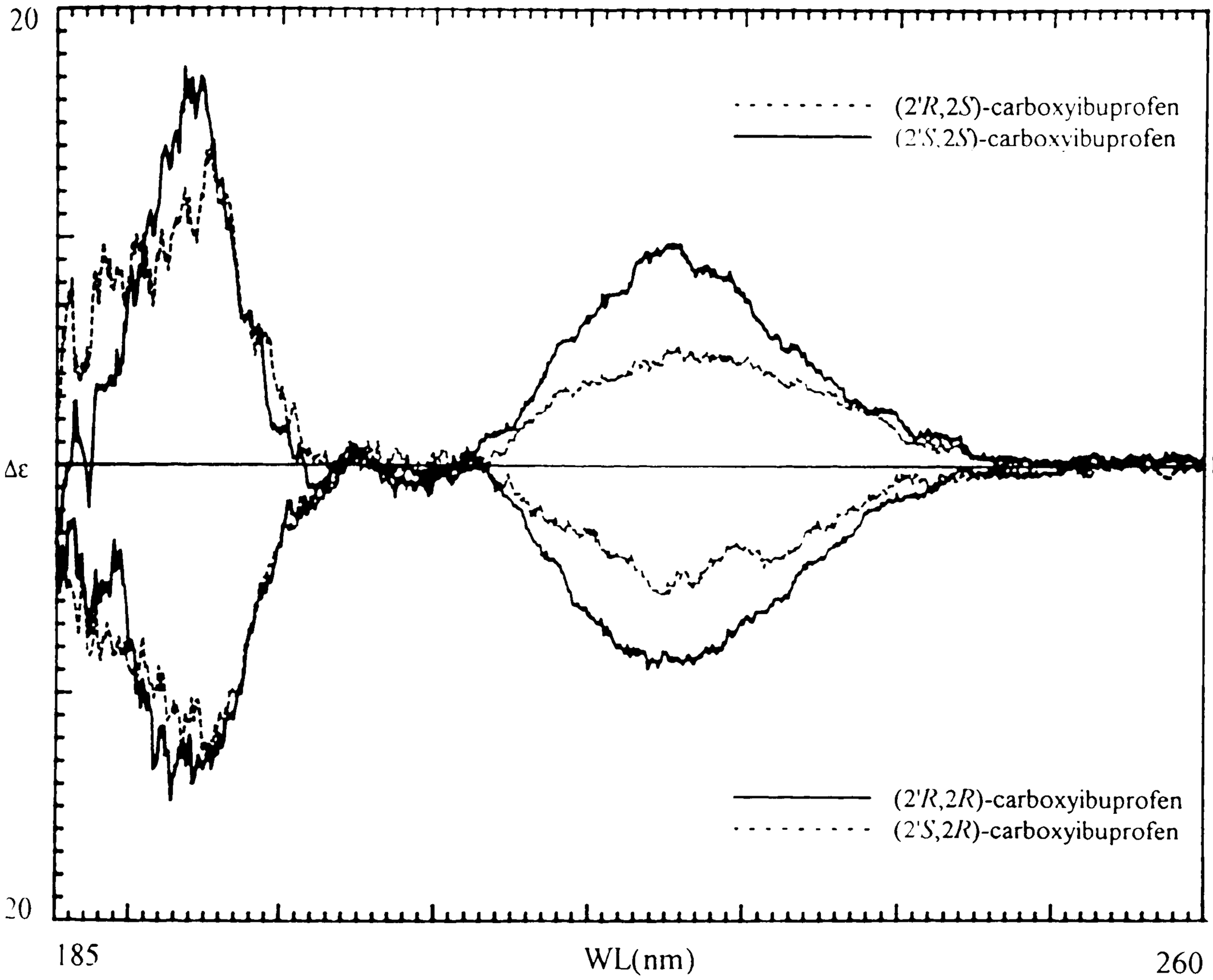


Figure 3.10: CD spectra presented from 185 to 260 nm for (2'*S*,2*R*)-, (2'*R*,2*R*)-, (2'*S*,2*S*)-, and (2'*R*,2*S*)-carboxyibuprofen. Samples dissolved (0.3 mg/ml) in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

the case of the 2'*R*,2*R*- stereoisomer, the contribution of the two chiral carbons with similar stereochemical configuration was additive, yielding a greater negative absorbance, as compared with (*R*)-ibuprofen. In the case of (2'*S*,2*R*)-carboxyibuprofen, the contribution of the two chiral centres are opposite. However, because the influence of the second chiral centre (2-carboxypropyl moiety) is weaker, the negative band is still obtained although the intensity is lower than that of (*R*)-ibuprofen. The converse is also true with (2'*S*,2*S*) and (2'*R*,2*S*)-carboxyibuprofen (Figures 3.9 and 3.10).

As (*R*)-2-methyl-3-phenylpropionic acid is structurally similar to the carboxy propyl moiety of carboxyibuprofen, examination of its CD spectrum would provide information on the relative effects of the two chiral centres on the CD spectrum of carboxyibuprofen. The CD spectrum reveals an absorbance band at 220 nm with a negative Cotton effect. Comparison of the CD spectra with that of (*R*)-ibuprofen showed that the Cotton effect observed was quantitatively weaker (Figure 3.11). Addition of the CD spectrum of this compound with that of (*R*)-ibuprofen yields a composite CD spectrum similar to that of (2'*R*,2*R*)-carboxyibuprofen (Figure 3.12), which supports the observation that the relative contribution of the carboxypropyl and propionic acid moieties towards the CD spectrum are additive. Similarly, spectral addition with that of (*S*)-ibuprofen yields a composite spectra resembling that of (2'*R*,2*S*)-carboxyibuprofen (Figure 3.13). In this case when the absolute configurations of the carboxypropyl and the propionic acid moieties are opposite the Cotton effects are also of opposite sign and hence give a weaker resultant band.

The CD spectra at the 260 nm region for (2'*R*,2*R*)- and (2'*S*,2*R*)-carboxyibuprofen were similar to that of (*R*)-ibuprofen and (*R*)-hydroxyibuprofen, reflecting the contribution of the ^1L_b transition and the effect of the stereochemical configuration arising from the propionic acid moiety and minimal influence of the second chiral centre carbon. The converse is also true for (*S*)-ibuprofen, (*S*)-hydroxyibuprofen and (2'*R*,2*S*)- and (2'*S*,2*S*)-carboxyibuprofen (Figures 3.14 and 3.15).

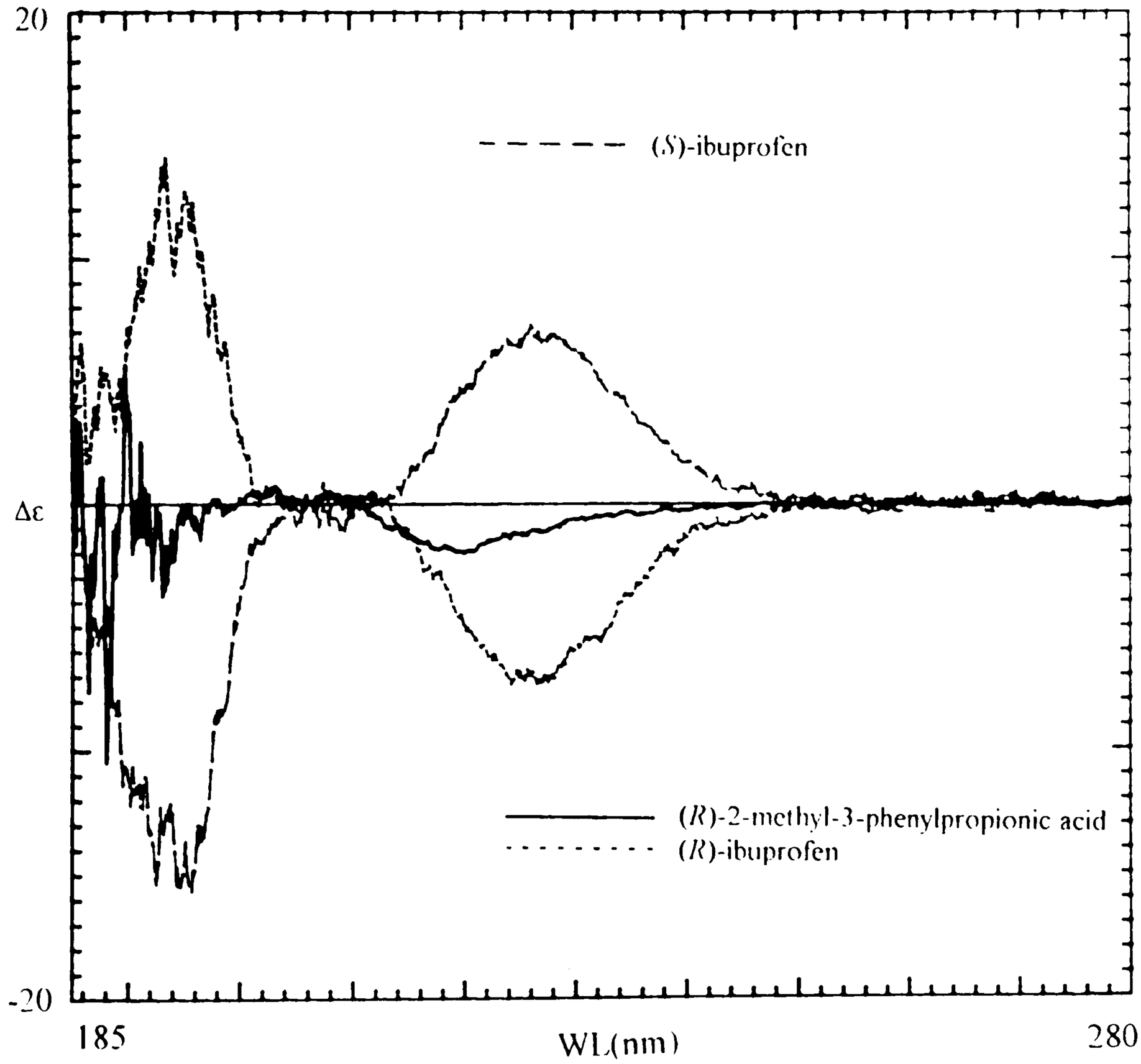


Figure 3.11: CD spectra presented from 185 to 280 nm for (*R*)-2-methyl-3-phenylpropionic acid, (*R*)- and (*S*)-ibuprofen. Samples dissolved (0.3 mg/ml) in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

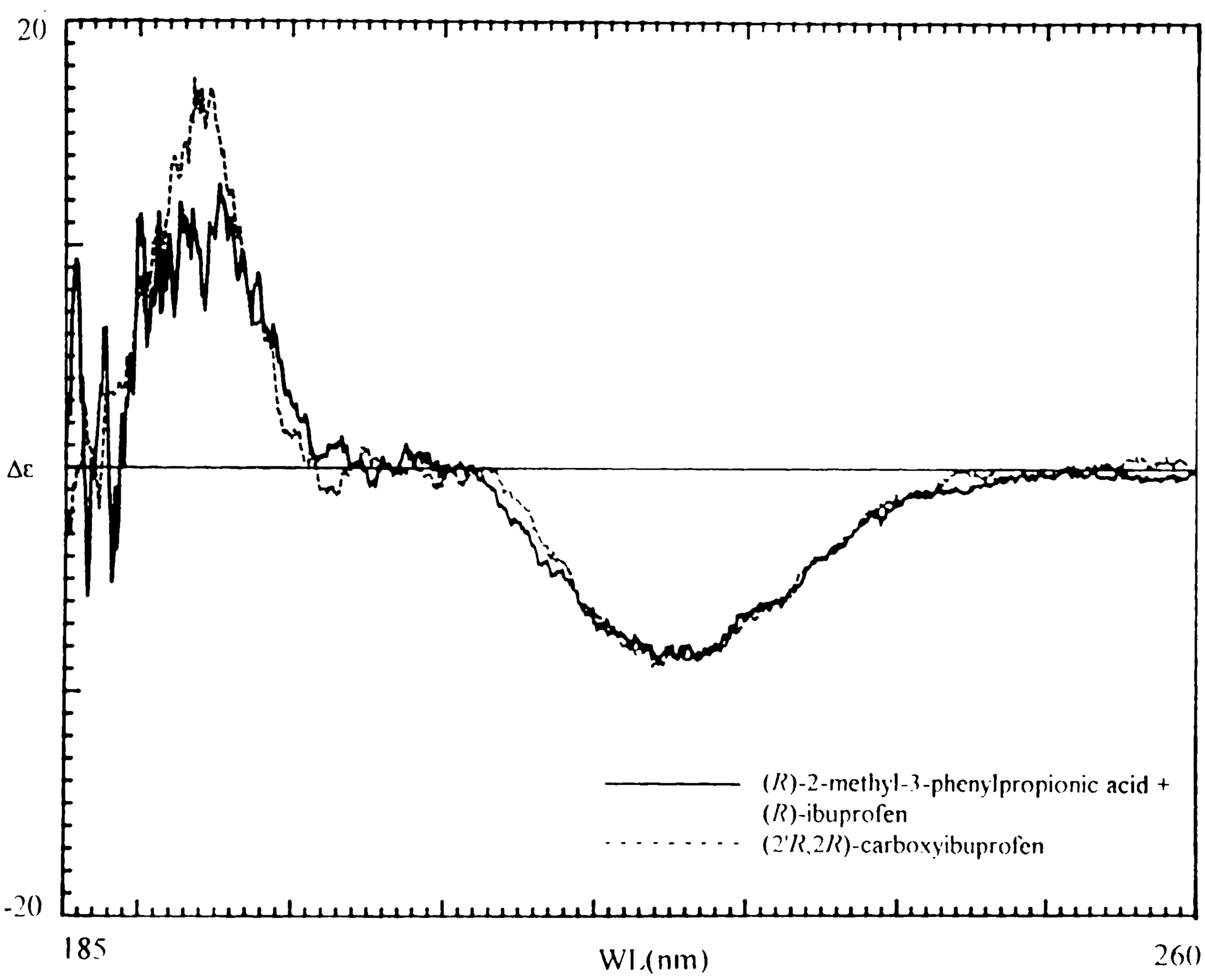


Figure 3.12: Composite CD spectrum presented from 185 to 260 nm after the addition of the CD spectrum of (*R*)-2-methyl-3-phenylpropionic acid and (*R*)-ibuprofen, overlaid with that CD spectrum for (2'*R*,2*R*)-carboxyibuprofen. Samples dissolved (0.3 mg/ml) in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

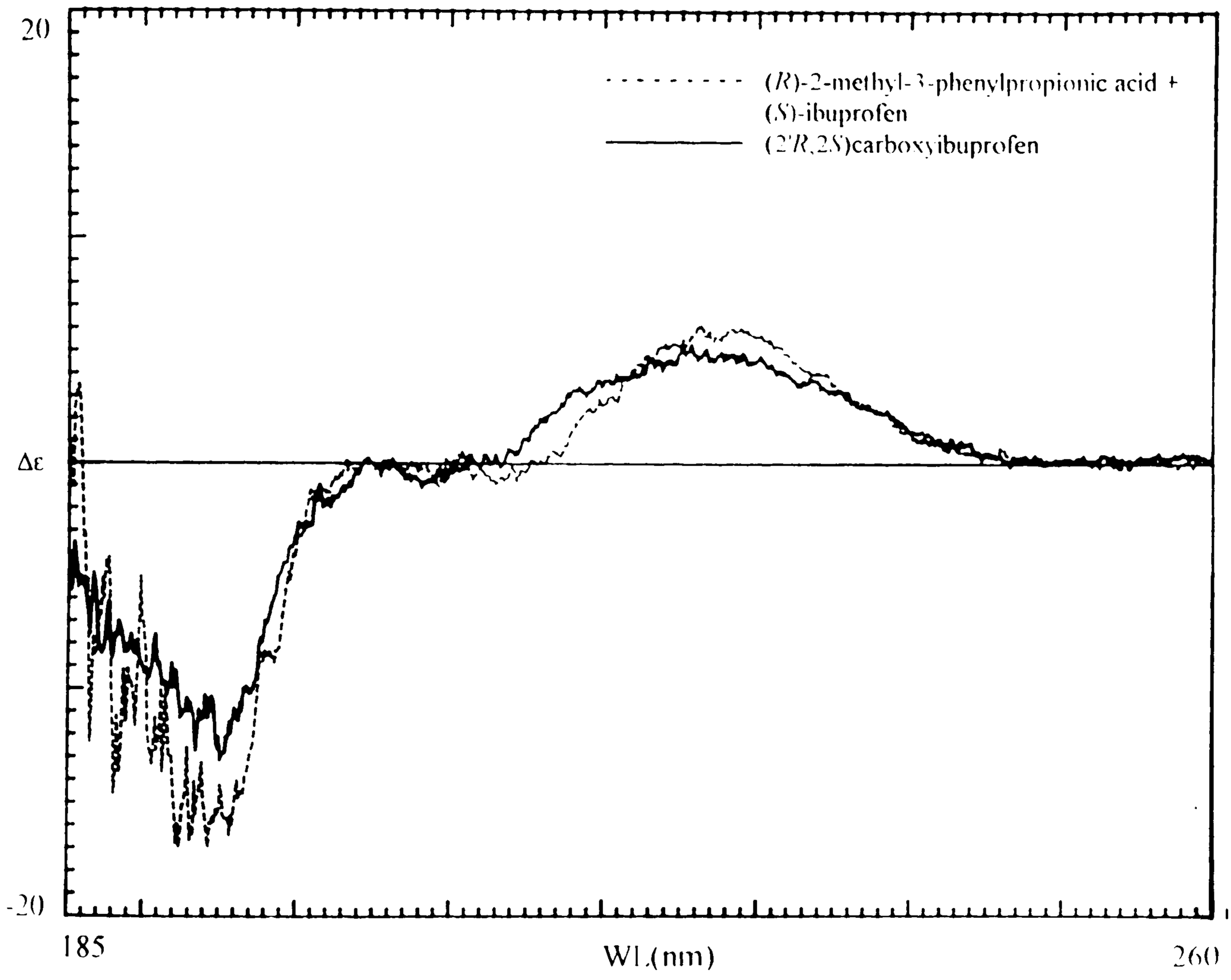


Figure 3.13: Composite CD spectrum presented from 185 to 260 nm after the addition of the CD spectrum of (*R*)- 2-methyl-3-propionic acid and (*S*)-ibuprofen, overlaid with that for (2'*R*,2*S*)-carboxyibuprofen. Samples dissolved (0.3 mg/ml) in acetonitrile; pathlength 0.02 cm; spectra recorded at 25°C.

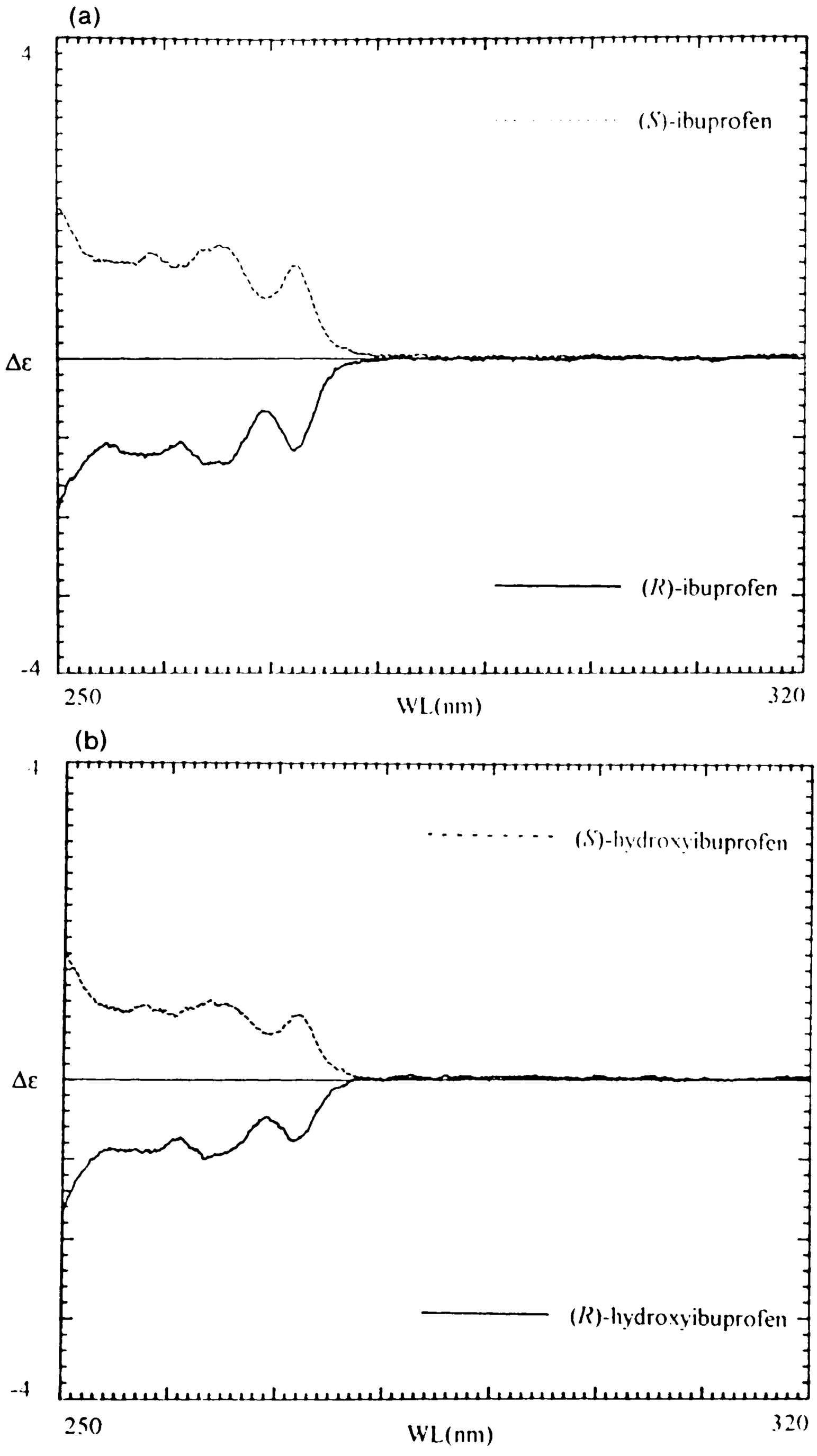


Figure 3.14: CD spectra presented from 250 to 320 nm for (a) (*R*)-ibuprofen (b) (*S*)-ibuprofen (c) (*R*)-hydroxyibuprofen and (d) (*S*)-hydroxyibuprofen. Samples dissolved (1.0 mg/ml) in acetonitrile; pathlength 1.0 cm; spectra recorded at 25°C.

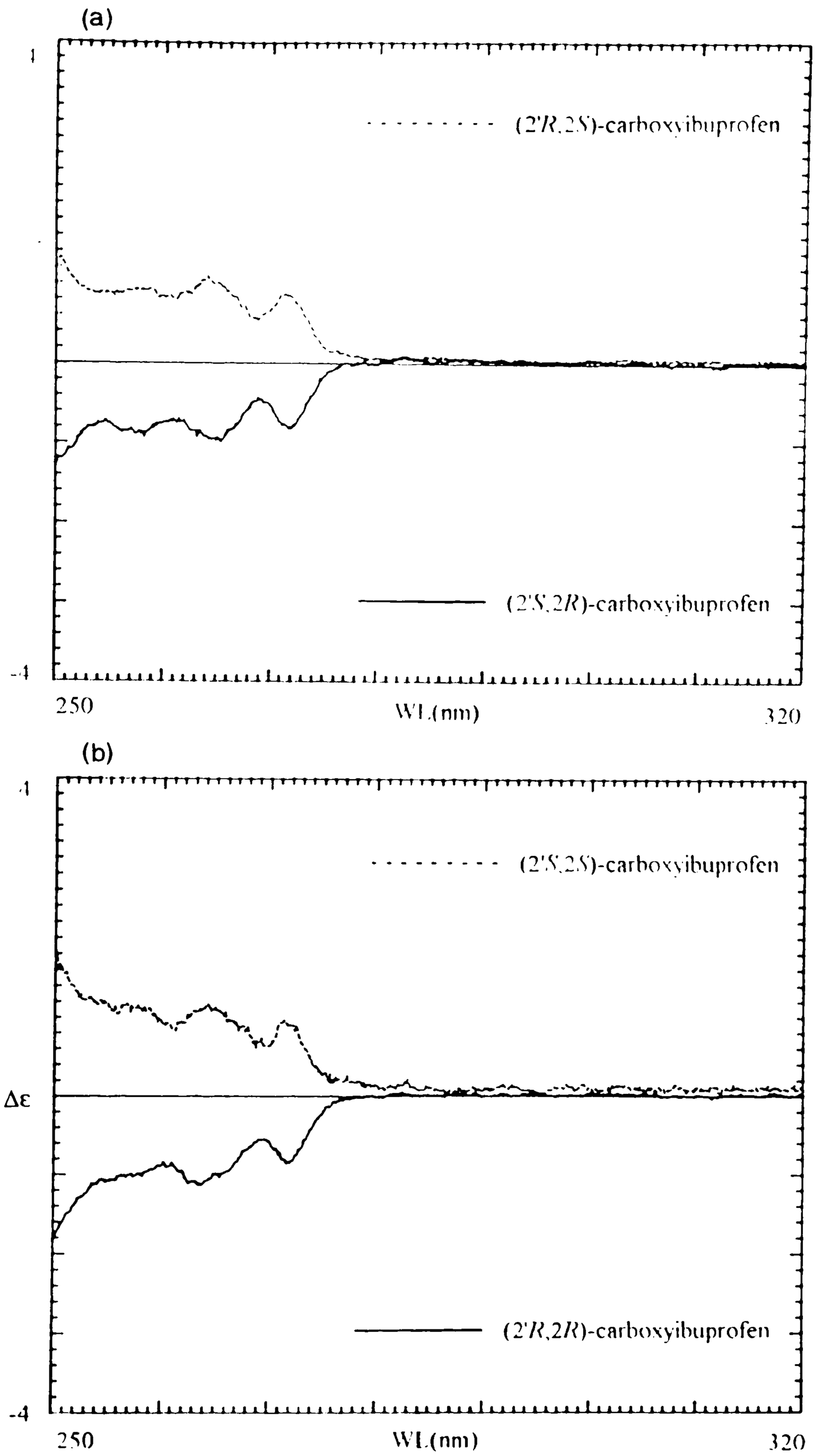


Figure 3.15: CD spectra presented from 250 to 320 nm for (a) (2'*S*,2*R*)- and (2'*R*,2*S*)-carboxyibuprofen (b) (2'*S*,2*S*)-carboxyibuprofen (2'*R*,2*S*)-carboxyibuprofen. Samples dissolved (1.0 mg/ml) in acetonitrile; pathlength 1.0 cm; spectra recorded at 25°C.

3.4 Conclusions

The carboxyibuprofen diastereoisomers were successfully resolved using a Chiralpak AD CSP and hexane:ethyl alcohol (92:8, v/v) containing TFA (0.05% v/v) as mobile phase. Analysis of the synthesised pairs of diastereoisomers, and urine obtained following the oral administration of 50 mg (*S*)-ibuprofen revealed that the elution order of the carboxyibuprofen diastereoisomers was 2'*S*,2*R*-, 2'*R*,2*R*-, 2'*R*,2*S*- and 2'*S*,2*S*- respectively. The CD spectra of the individual diastereoisomers were compared with those of both (*R*)- and (*S*)-ibuprofen, as well as (*R*)- and (*S*)-hydroxyibuprofen. Generally the CD spectra for the respective enantiomers of ibuprofen and hydroxyibuprofen were similar. With the carboxyibuprofen diastereoisomers, major differences were seen only in the 225 nm band. The effect of the second chiral centre in the CD spectra were similar, although quantitatively weaker in intensity than that of the original chiral centre with the same stereochemical configuration. Thus, with the 2'*R*,2*R*- and 2'*S*,2*S*- diastereoisomers, stronger bands were observed at this region as compared with (*R*)- and (*S*)-ibuprofen respectively. When the second chiral centre is of opposite configuration, the intensity of this band is attenuated, although the direction is still the same, reflecting the weaker contribution of the second chiral centre. Consistent with the above observations, the CD spectra of (*R*)-2-methyl-3-phenylpropionic acid when combined with that of (*R*)-ibuprofen and (*S*)-ibuprofen yields CD spectra similar to that of (2'*R*,2*R*)-carboxyibuprofen and (2'*R*,2*S*)-carboxyibuprofen respectively.

The chromatographic system employed above is the first to resolve all four stereoisomers of carboxyibuprofen. The application of this methodology to the development of a sequential chromatographic technique for the determination of the stereochemical composition of the metabolites in urine is described in the next chapter.

CHAPTER 4

Stereospecific Analysis of Carboxyibuprofen and Hydroxyibuprofen in Urine

4.1 Introduction

Compared with the large number of published methods for the determination of ibuprofen enantiomers in biological fluids (see Chapter 2 and references therein), there are relatively few methods for the analysis of carboxyibuprofen and hydroxyibuprofen (Lockwood and Wagner, 1982; Shah and Jung, 1986; Chai *et al.*, 1988; Geisslinger *et al.*, 1989), or that of their enantiomers (see below), in urine. This is quite surprising considering the significant interest in the stereoselective processes of ibuprofen metabolism. The reason for this is probably due to methodological difficulties presented as a result of the introduction of a second chiral centre in the carboxy metabolite, coupled with the lack of authentic standards of the individual isomers. An important pre-requisite for such metabolic studies is that specific, sensitive and reproducible analytical methods must be developed to quantitate the individual isomers of both metabolites in urine. The enantiospecific analysis of (*R*)- and (*S*)-hydroxyibuprofen can be easily achieved by a variety of methods (Kaiser *et al.*, 1976; Young *et al.*, 1986; Baillie *et al.*, 1989; Rudy *et al.*, 1990; Chen and Chen, 1994). However, it is the separation of the carboxyibuprofen diastereomers that presents the biggest hurdle. A major problem with the analysis of the dicarboxylic acid metabolite being the lack of resolution two of the stereoisomeric metabolites (Kaiser *et al.*, 1976; Young *et al.*, 1986; Nicoll-Griffith *et al.*, 1988; Baillie *et al.*, 1989). Following the stereochemical assignment and chromatographic separation of the individual isomers of carboxyibuprofen as described in Chapter 3, this methodology is now developed into a sequential normal-chiral phase method for the stereospecific determination of hydroxy and carboxyibuprofen in urine. The normal-phase assay quantitates the total concentration of the individual hydroxy and carboxy metabolites in urine, the eluate from the column containing carboxy and hydroxyibuprofen are separately collected, dried and injected onto the chiral stationary phase for the determination of the stereochemical composition of the metabolites.

4.2 Experimental

4.2.1 Chemicals and Reagents

Dichloromethane, ethanol, ethyl acetate and hexane (HPLC grade) were purchased from Rathburn (Walkerburn, U.K.). Trifluoroacetic acid, sodium dihydrogen phosphate and sodium hydroxide (Analar grade) and other organic solvents (GPR grade) were obtained from BDH (Poole, Dorset, U.K.). Hexamethyldisilazane and 4-chlorophenoxyacetic acid were obtained from Sigma Chemicals (Poole, Dorset, U.K.). (*R,S*)-, (*R*)- and (*S*)-hydroxyibuprofen were a gift from Boots Ltd. (Nottingham, U.K.). "Racemic" carboxyibuprofen was synthesised by Dr. J.A. Baker of University of Brighton, using the procedure outlined in Appendix 2.

4.2.2 Chromatographic Columns and Supplies

The Partisil silica column (250 x 4.6 mm, 5 µm) was obtained from Whatman (Maidstone, Kent, U.K.). Refillable guard columns (10 x 2.1 mm) were packed with pellicular silica (40-63 µm), both obtained from Alltech (Lancs, U.K.). The chiral column a Chiralpak AD (amylose tris(3,5-dimethylphenylcarbamate)) column (250 x 4.6 mm, 10 µm) was used with a matching guard column (5 x 4.6 mm, 10 µm), was supplied by HPLC Technology Ltd. (Macclesfield, U.K.). Merck GF_{254} TLC plates were obtained from BDH (Poole, Dorset, U.K.).

4.2.3 Instrumentation

Normal phase HPLC was performed using a LDC Constametric 3000 pump, a LDC Spectromonitor 3100 UV detector and a LDC CI4000 computing integrator (Stone, Staffs). Samples were injected using a LKB 2157 autosampler (Pharmacia, Ltd, Milton Keynes). Chiral-phase HPLC was performed using a LDC Constametric 3000 pump, a LDC Spectromonitor 3100 UV detector and a LDC CI4100 computing

integrator (Stone, Staffs). Samples were injected using a Perkin Elmer ISIS 100 autosampler (Beaconsfield, Bucks, U.K.).

4.2.4 Preliminary Thin Layer Chromatography (TLC) Experiments

Thin Layer chromatography was used to obtain preliminary solvent conditions prior to normal-phase HPLC. Aluminium backed TLC plates were cut into 3 x 10 cm strips. At a distance of 1.0 cm from the bottom edge of each strip were spotted *ca.* 1 µg amounts of (*R,S*)-hydroxyibuprofen, racemic carboxyibuprofen and blank hydrolysed urine extracted with diethylether (see section 2.2.6). The strips were separately developed using the following solvents: hexane, toluene, dichloromethane, ethyl acetate, chloroform and isopropyl alcohol, to determine the elution strength of the solvents with respect to the analytes. The plates were developed for a distance of 8 cm and spots were located under a short-wave UV light source. Based on these experiments, various mixtures of organic solvents were used in order to obtain R_f values of 0.3 to 0.6 for the analytes, and trifluoroacetic acid (0.1%) was added to the solvent mixtures to obtain more defined spots and reduce streaking. These 'scouting' experiments provided preliminary solvent conditions for normal-phase HPLC.

4.2.5 Development of an extraction method for the analysis of Carboxyibuprofen and Hydroxyibuprofen in urine.

a) Silanization of glassware

All glass tubes used in the extraction procedure were silanized prior to use. This was done by rinsing glass tubes with a solution of hexamethyldisilazane (10%, v/v) in dry toluene. The glass tubes were then placed in a hot air oven at 100^{0}C for one hour. The tubes were then rinsed with methanol and further dried in the oven. The same procedure was also carried out for the glass pasteur pipettes used in extraction procedure.

b) Solvent Extraction

For the determination of unconjugated metabolites, 0.5 ml aliquots of urine were used. To these samples were added 25 µg (50 µl of a 0.5 mg/ml solution in acetonitrile) of p-chlorophenoxyacetic acid as internal standard. The samples were then acidified by adding of hydrochloric acid (1.0M; 100µl) and buffered to pH 3.8 with 1.5 ml of phosphate buffer (pH 3.8, 1.0M). Dichloromethane:ethyl acetate (14:1, v/v; 5 ml) was added and the extraction tubes were then tightly capped and mixed on a test-tube rocker for 20 minutes. Phase separation was achieved by centrifugation for 5 minutes at 1000g. The lower organic layer was then separated into a clean glass tube and evaporated under a gentle stream of nitrogen at 40° C on a dry heating block. The residue was then reconstituted in 150 µl mobile phase and 50µl was injected into the achiral HPLC system.

For the determination of conjugated metabolite concentrations, 0.1 ml aliquots of urine were used. To these samples were added 25 µg (50 µl of a 0.5 mg/ml solution in acetonitrile) of p-chlorophenoxyacetic acid and NaOH (1.0M; 20µl). The hydrolysis reaction was left to proceed for 2 hours at room temperature, at the end of which HCl (1.0M; 40 µl), phosphate buffer (pH 3.8, 1.0M; 200 µl) and dichlromethane:ethyl acetate (14:1, v/v; 1 ml) as extraction solvent were added The tubes were mixed and extraction carried out as before. All analysis of urine samples were done in duplicate.

c) Chromatographic conditions

The column used was a Partisil 5 silica column protected by a guard column filled with pellicular silica. The mobile phase consisted of hexane:ethanol (98.2:1.8, v/v) with 0.05% trifluoroacetic acid as modifier, run at a flow rate of 2 ml/min. The UV detector was set at 220 nm and the detector output was set at 0.02 a.u.f.s.

4.2.6 Validation of the normal-phase HPLC procedure

Stock solutions of "racemic" carboxyibuprofen and hydroxyibuprofen (1 mg/ml) were prepared in acetonitrile. Into six separate silanized volumetric flasks (10 ml) were pipetted 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 ml of each solution. The solutions were evaporated gently under nitrogen gas. Blank drug-free urine was added to the flasks *q.s.* to 10 ml to give final solutions of 10, 20, 40, 80, 160 and 320 µg/ml of each metabolite respectively. On each day of analysis, 0.5 ml of these solutions were pipetted out into separate tubes and analysed. Calibration curves were constructed by plotting peak area ratios (metabolite:internal standard) against concentration of the metabolite concerned and subjecting this data to linear regression analysis. The concentration of urine and spiked samples were determined by comparing measured peak area ratios to the calibration curve prepared.

The accuracy and within day variation of the assay was assessed by analysing six "spiked" samples each of the following calibration samples: 10, 80 and 320 µg/ml. The precision and accuracy of the assay was determined for each metabolite by calculation of the percentage coefficient of variation, CV% (S.D./mean x 100%) and the mean percentage difference (mean concentration - actual concentration / actual concentration x 100%). The recovery of the extraction procedure was determined by comparing the peak areas obtained with direct injections of standard solutions of the same concentration (n = 6).

The between day variation of the assay was determined by analysing calibration urine samples of 10, 80 and 320 µg/ml concentration for 6 consecutive days. The coefficient of variation and accuracy were calculated as before.

4.2.7 Fraction collection

The eluate from the HPLC containing carboxy (R_t, 12.6 min; fraction collected between 12.2 and 13.0 min) and hydroxyibuprofen (R_t, 15.8 min; fraction collected between 15.3 and 16.3 min) were separately collected from the detector outlet into silanized tubes. The eluent was gently evaporated under nitrogen gas at 40° C in a dry block heater and the residue reconstituted in 100 µl mobile phase and subjected to chiral analysis. In order to selectively collect the peaks of interest, the outlet tube from the detector was shortened as much as possible (10 cm) to reduce the lag time between detector response and the actual elution of the analyte from the detector outlet.

4.2.8 Chiral-phase analysis of carboxyibuprofen and hydroxyibuprofen in urine.

Standard metabolite solutions dissolved in mobile phase or eluate collected from the normal-phase analysis redissolved in the mobile phase used for chiral chromatography were injected in 50 µl aliquots onto the HPLC. The mobile phase was hexane:ethanol (92:8, v/v) with trifluoroacetic acid (0.05%, v/v) as modifier, run at a flow rate of 1 ml/min. The detection wavelength was set at 220 nm. The column used was a Chiralpak AD column (250 x 4.6 mm, 10 µm) connected to a guard column containing similar material (5 x 4.6 mm, 10µm).

The enantiomeric composition of each enantiomer of hydroxyibuprofen was determined as follows:

$$\text{Enantiomeric composition} = \frac{\text{Peak area of enantiomer}}{\text{Total peak area of both enantiomers}}$$

Individual concentrations of each enantiomer were calculated by multiplying their enantiomeric composition by the corresponding metabolite concentrations obtained from the normal-phase analysis.

For carboxyibuprofen, the same approach was used, the total peak area being for all four stereoisomers.

4.2.9 Validation of the chiral assay procedure.

In order to determine whether the chiral HPLC method could produce accurate stereochemical compositions over a wide range of concentrations, the HPLC eluate from the validation experiments in section 4.2.6 were collected and subjected to chiral phase analysis. Enantiomeric ratios were determined for both metabolites and the coefficient of variation and mean percentage difference calculated.

4.3 Results and Discussion

When CSPs are used in bioanalysis, separation problems are often encountered due to the restricted choice of solvent conditions available. Also the presence of co-extracted contaminants may cause problems in analyte retention, resolution and column stability (Camillieri *et al*, 1994). Some of these problems associated with CSPs can be overcome by using coupled column chromatography, where drug concentrations are determined on an achiral phase and the appropriate fraction of the eluate transferred to a CSP via a switching valve to determine enantiomeric compositions (Oda *et al.*, 1992). In our situation, the use of this technique seems relevant as the mobile phases used are compatible. However, a two step approach is adopted to obtain a high throughput of samples and to increase assay sensitivity in the chiral separation stage by concentrating the collected eluate before introduction into the CSP.

4.3.1 Normal-phase analysis of carboxyibuprofen and hydroxyibuprofen in urine

The choice of normal-phase chromatography over reversed-phase chromatography for achiral analysis was based on the fact that the fractions collected would be more easily evaporated. Had reversed-phase chromatography been used, the aqueous solvents would have to be freeze-dried. This would probably result in substantial loss in the amount of metabolite recovered, and subsequently cause sensitivity problems with the chiral analysis. Besides, salt residues from the buffer used in reversed-phase analysis may affect chiral-phase stability.

The use of TLC as a preliminary method for obtaining initial solvent conditions prior to normal-phase LC proved quite successful. From initial solvent compositions of hexane:ethanol and hexane:isopropanol (9.5:0.5, v/v) containing trifluoroacetic acid (0.1% v/v), it was apparent that the hexane:ethanol mixture gave better separation between carboxyibuprofen and hydroxyibuprofen. Solvent composition was finally optimised at hexane:ethanol (98.2:1.8, v/v) and the proportion of trifluoroacetic acid was fixed at 0.05% v/v as the percentage of modifier did not appear to have much influence on either analyte retention or resolution at concentrations of 0.05 to 0.1 % v/v. Under the conditions used, the retention times of the internal standard, carboxyibuprofen and hydroxyibuprofen were 4.9, 12.6 and 15.8 minutes respectively (Figure 4.1). The resolution factor between the latter two peaks are calculated to be 1.5.

UV Detection was set at 220 nm to increase sensitivity as the phenylpropionic moiety has low molar absorptivity at higher wavelengths. Although it was realised that the shorter wavelength could potentially cause difficulties when measuring extracts from urine due to high background noise from endogenous materials, with a careful choice of sample preparation conditions and the relatively high concentrations of analytes in urine, these problems were avoided. Also, the

major constituents of the mobile phase are hexane and ethanol, both of which have relatively good UV transmittance at 220 nm, thus increasing assay sensitivity.

4.3.2 Extraction procedure for carboxyibuprofen and hydroxyibuprofen in urine

A chromatogram showing the clean chromatographic baselines achieved with blank hydrolysed urine following treatment with sodium hydroxide is shown in Figure 4.1. No interferences were seen at the retention times of either carboxyibuprofen and hydroxyibuprofen at the level of sensitivity used. The choice of dichloromethane:ethyl acetate (14:1, v/v) was arrived at after much experimenting with various solvents. Dichloromethane gave the cleanest extracts but recovery was only 40% with a single 5 ml extraction. This is consistent with the observation by Rudy *et al.*,(1990) where a 2 x 7 ml extraction procedure was used. Mixtures of dichloromethane and ethyl acetate were experimented with and it was found that a proportion of 14:1 v/v was found to give good recoveries (Tables 4.1 and 4.2) with low background noise.

For the hydrolysis of conjugated metabolites, the urine samples are usually hydrolysed either by acid (Nicol-Griffith *et al.*, 1988; Chai *et al.*, 1988 and Rudy *et al.*, 1990) or by base (Lockwood and Wagner, 1983; Baillie *et al.*, 1989; Geisslinger *et al.*, 1989) under mild conditions in order to avoid racemization. Here the procedure of base hydrolysis for the cleavage of the ester glucuronides was essentially similar to that used for the determination of ibuprofen in urine.

4.3.3 Validation of the normal-phase method.

The analytical procedure showed good precision and accuracy while extraction recoveries were generally better than 90% for both metabolites at all concentration levels (Tables 4.1 and 4.2). The day to day variation data also showed

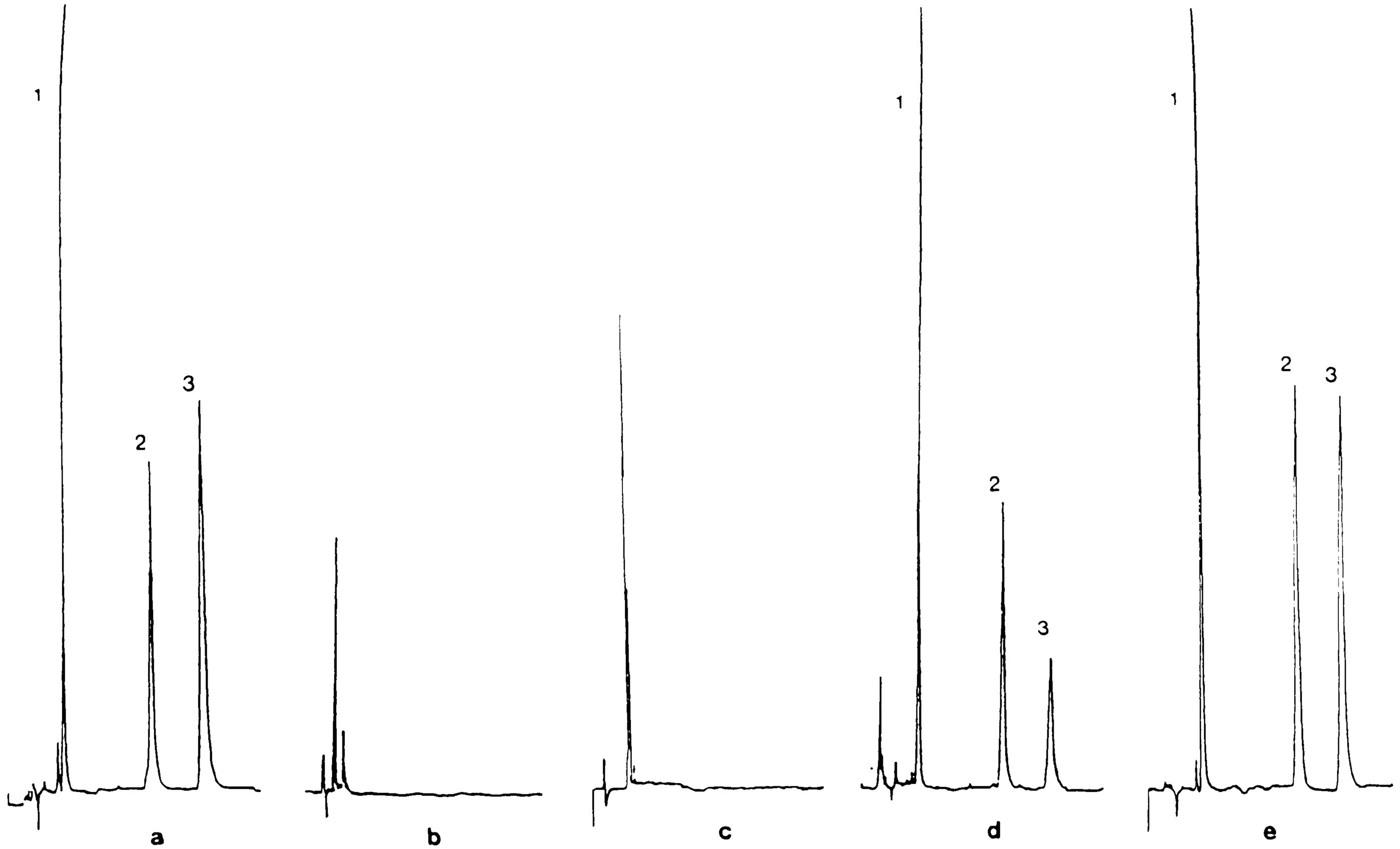


Figure 4.1: Normal-phase chromatograms of (a) a standard solution containing 100 µg/ml of p-chlorophenoxyacetic acid, peak 1: 4.9 mins and 180 µg/ml each of carboxyibuprofen, peak 2: 12.6 mins (α= 2.64, R_s = 3.2) and hydroxyibuprofen, peak 3: 15.8 mins (α = 1.85, R_s = 3.3), (b) blank urine extract, (c) alkali treated blank urine extract, (d) extract of a urine sample and (e) alkali treated urine sample from a volunteer 2-4 hr after oral administration of 400 mg racemic ibuprofen.

Table 4.1: (a) Within day variation, accuracy and extraction recoveries of carboxyibuprofen in "spiked" urine samples (n=6) and (b) between day variation and accuracy of carboxyibuprofen in spiked urine samples (n=6).

(a)

Concentration (µg/ml)	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference	Recovery (%)
10	10.22 ± 0.33	3.2	2.16	94.2 ± 3.0
80	79.94 ± 0.46	0.6	-0.08	93.1 ± 0.5
320	322.9 ± 11.82	3.7	0.93	90.5 ± 3.3

(b)

Concentration (µg/ml)	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference
10	9.66 ± 0.68	7.0	-3.40
80	82.68 ± 6.67	8.1	3.36
320	325.7 ± 30.9	8.0	1.79

Table 4.2: **(a) Within day variation, accuracy and extraction recoveries of hydroxyibuprofen in "spiked" urine samples (n=6) and (b) between to day variation and accuracy of hydroxyibuprofen in spiked "urine" samples (n=6).**

(a)

Concentration (µg/ml)	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference	Recovery (%)
10	10.24 ± 0.70	6.8	2.36	96.6 ± 6.6
80	79.85 ± 2.23	2.8	-0.18	97.1 ± 2.7
320	320.7 ± 16.8	5.2	0.21	93.2 ± 4.9

(b)

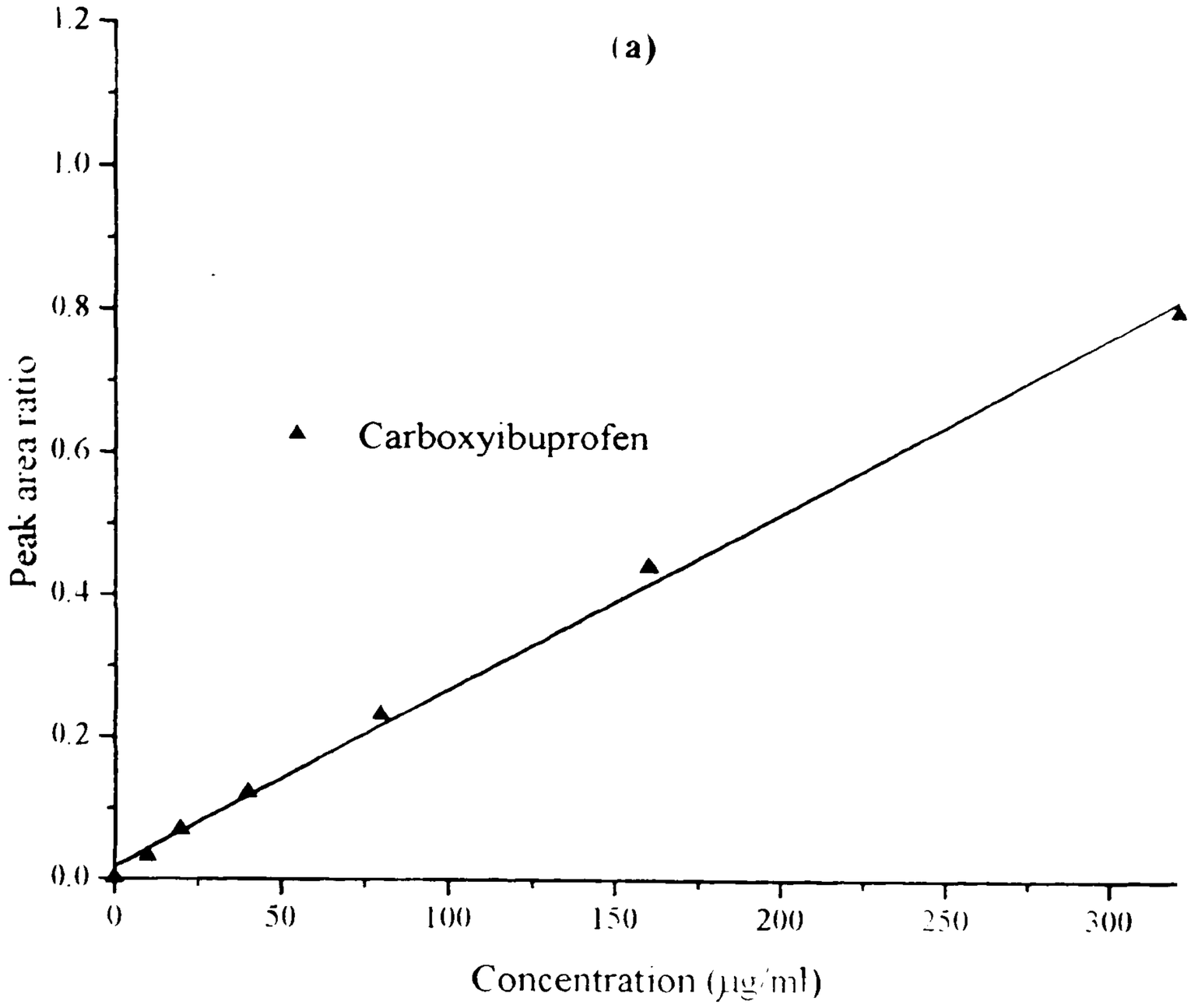
Concentration (µg/ml)	Concentration Determined (µg/ml)	Coefficient of Variation (CV%)	Mean Percent Difference
10	9.91 ± 0.70	7.0	-0.92
80	80.37 ± 4.32	5.4	0.46
320	314.5 ± 30.7	9.8	-1.73

good consistency and accuracy (Tables 4.1 and 4.2). Typical calibration curves for both metabolites are shown in Figure 4.2. Linear regression analysis showed regression coefficients of better than 0.997. The limit of quantitation for the procedure was at least 10 µg/ml urine. A lower limit of detection can be easily achieved by analysing a larger volume of urine or by injecting a larger proportion of the extract on-column. However, this is not necessary in this case as the quantity of analytes in urine resulting from a typical oral dose of ibuprofen is relatively high.

4.3.4 Chiral separation of carboxyibuprofen and hydroxyibuprofen enantiomers

The HPLC conditions used for the chiral analysis were similar to those used in Chapter 3 for the initial resolution of the stereoisomers of carboxyibuprofen. Under these conditions the retention times of the 2'*S*,2*R*-, 2'*R*,2*R*-, 2'*R*,2*S*- and 2'*S*,2*S*- isomers of carboxyibuprofen were at 11.0, 12.1, 16.9 and 20.1 minutes respectively. (Figure 4.3). The resolution factors for the four peaks were 1.3, 3.3 and 2.4 respectively (Figure 4.3), indicating very good baseline separation, when compared to work published previously (Kaiser *et al*, 1976; Baillie *et al.*, 1989 and Rudy *et al.*, 1990). The same mobile phase conditions were also used for the analysis of hydroxyibuprofen. The elution order of the enantiomers was confirmed by injection of authentic enantiomers. Here the *R*-enantiomer eluted before its *S*-antipode, with retention times of 13.9 and 16.1 minutes respectively. The separation was baseline with a resolution factor of 1.6 (Figure 4.4).

The sequential normal-chiral phase approach, although tedious, proved to be rather successful. As can be seen from the blank urine eluate, the collected fractions were generally free from interferences. Had the urinary extracts been injected directly into the chiral phase, there would be co-elution of (*R*)-hydroxyibuprofen with (2'*R*,2*R*)-carboxyibuprofen and (*S*)-hydroxyibuprofen with (2'*R*,2*S*)-carboxyibuprofen under the conditions used. Although these can eventually be separated by using hexane:ethanol:methanol (95:3.5:1.5, v/v) with trifluoroacetic acid (0.05%, v/v) as modifier, the resolution of the six peaks would be considerably



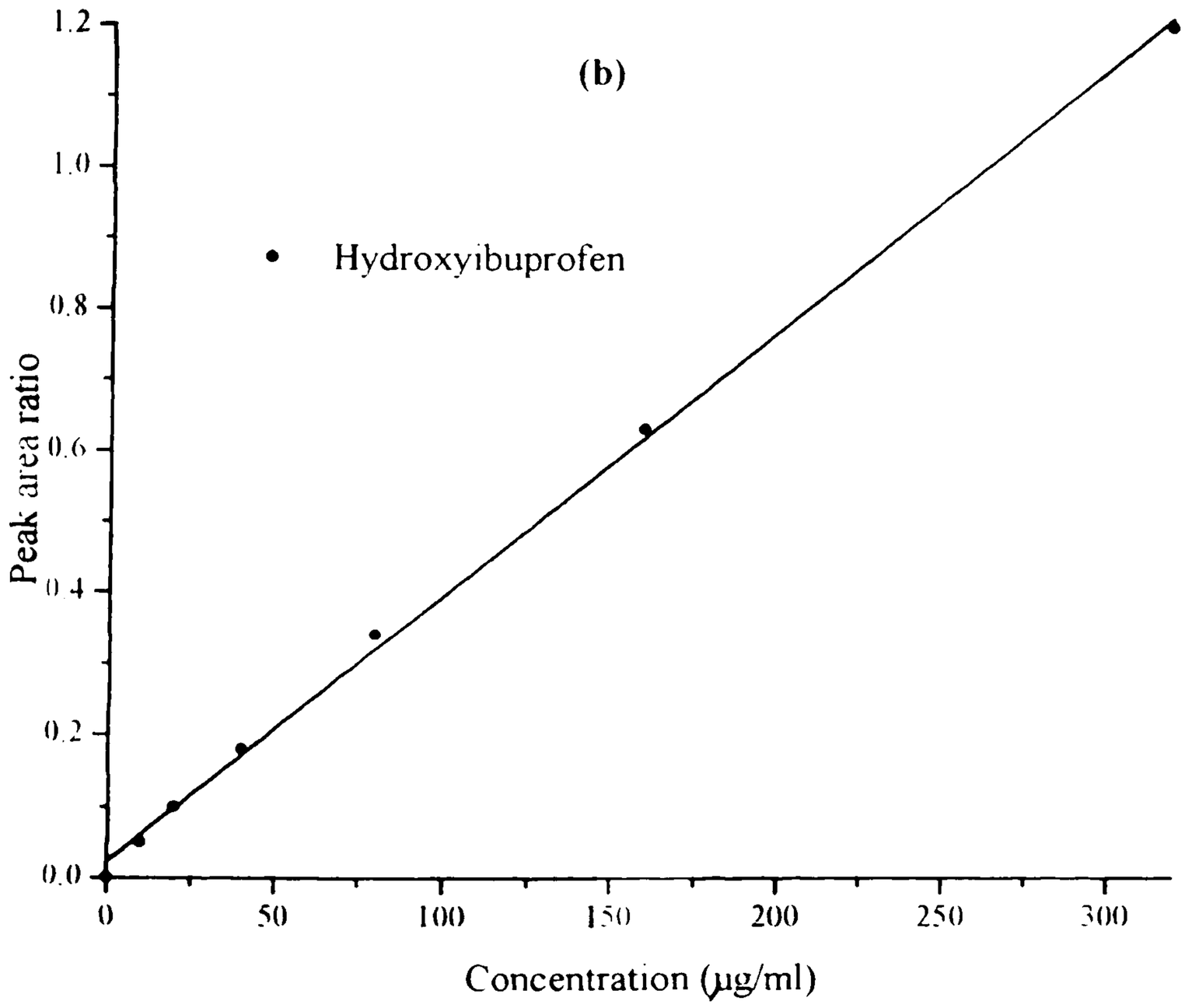


Figure 4.2: Typical calibration curves prepared from the quantitation of (a) carboxyibuprofen and (b) hydroxyibuprofen using the normal phase HPLC assay.

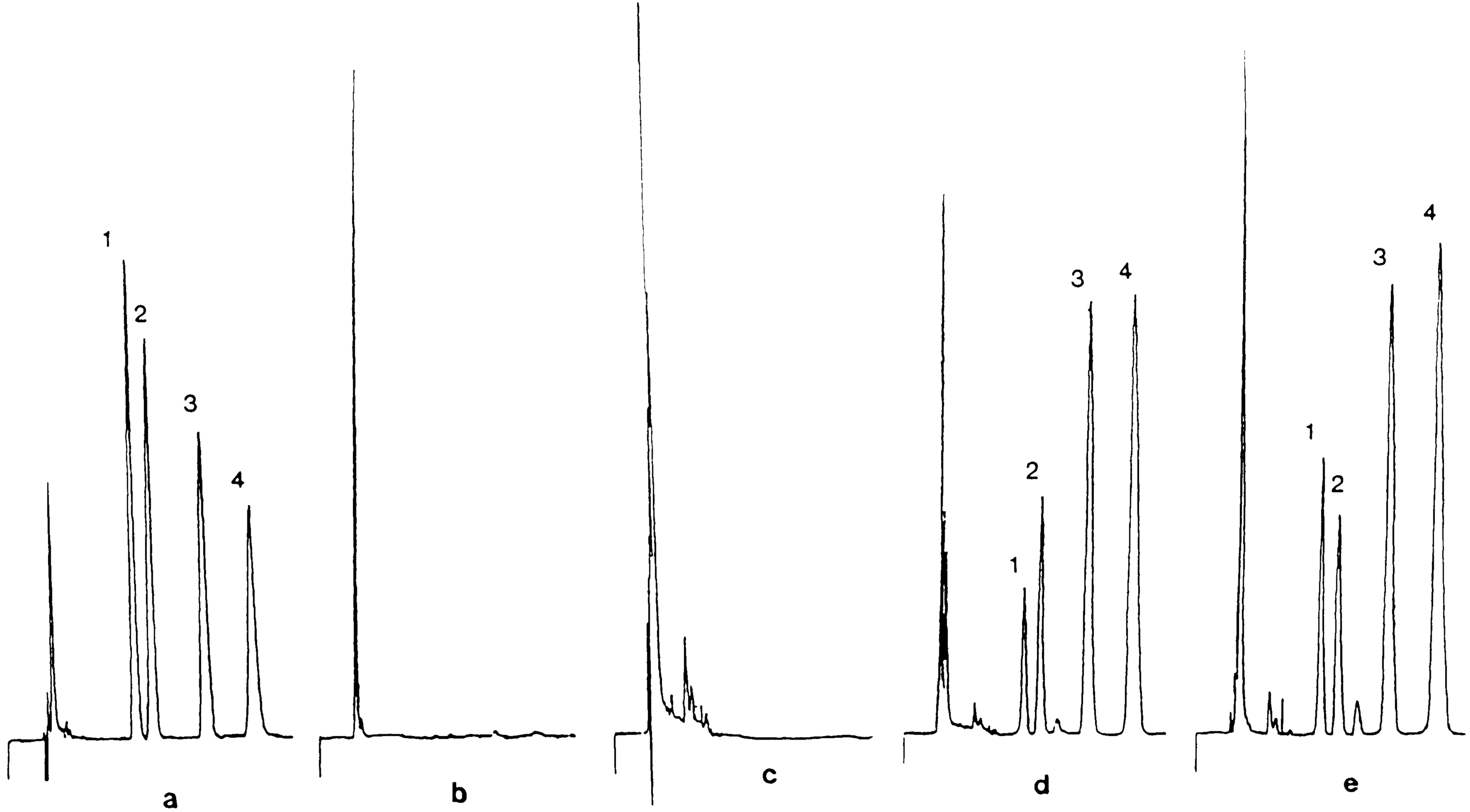


Figure 4.3: Chiral-phase chromatograms of (a) a standard solution of "racemic" carboxyibuprofen solution (80 μg/ml). Retention times of diastereomers ,peak 1, (2'*S*,2*R*)- : 11.0 min; peak 2 (2'*R*,2*R*): 12.1 min (α = 1.20, R_s = 1.3); peak 3 (2'*R*,2*S*)-: 16.9 min (α = 1.5, R_s = 3.3); peak 4(2'*S*,2*S*)-: 20.1 min (α = 1.3, R_s = 2.4), (b) eluate from blank urine analysed by normal-phase HPLC, (c) eluate from a alkali treated blank urine analysed by normal-phase HPLC, (d) eluate from the normal-phase analysis of urine and (e) alkali treated urine from a volunteer 2-4 hr after oral administration of 400 mg racemic ibuprofen [Mobile phase, hexane:ethanol (92:8,v/v) with TFA (0.05%); Flow rate, 1.5 ml/min; Detection, UV λ = 220 nm].

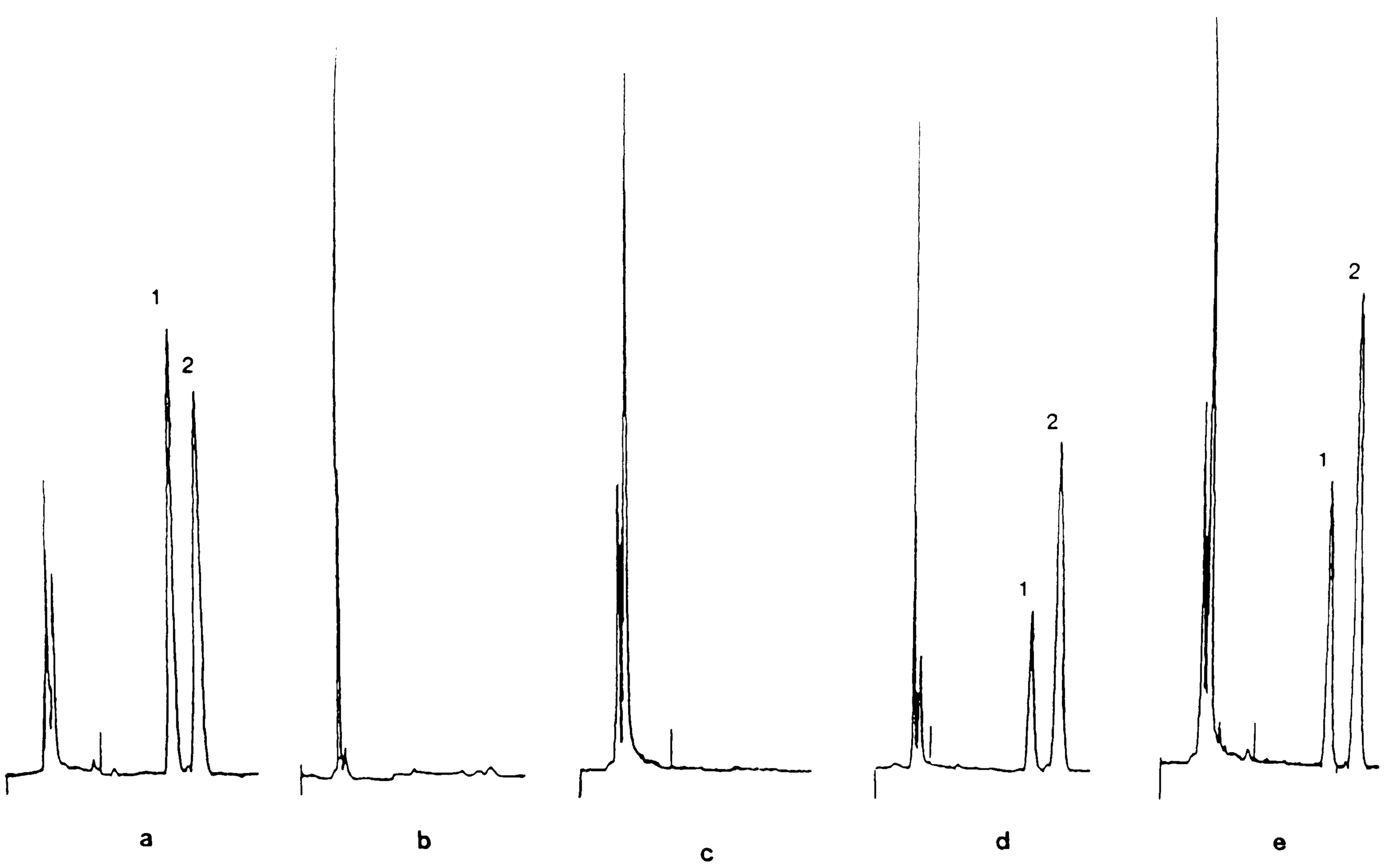


Figure 4.4: Chiral-phase chromatograms of (a) a standard solution of racemic hydroxyibuprofen, 80 µg/ml. Retention times, peak 1 (*R*)-hydroxyibuprofen): 13.9 min; peak 2 ((*S*)-hydroxyibuprofen): 16.1 min (α = 1.25, R_s = 1.6), (b) eluate from a blank urine analysed by normal-phase HPLC, (c) eluate from alkali treated blank urine analysed by normal-phase HPLC, (d) eluate from the normal-phase analysis of urine and (e) alkali treated urine from a volunteer 2-4 hr after oral administration of 400 mg racemic ibuprofen. [Mobile phase, hexane:ethanol (92:8, v/v) with TFA (0.05%, v/v); Flow rate, 1 ml/min; Detection, UV λ = 220 nm].

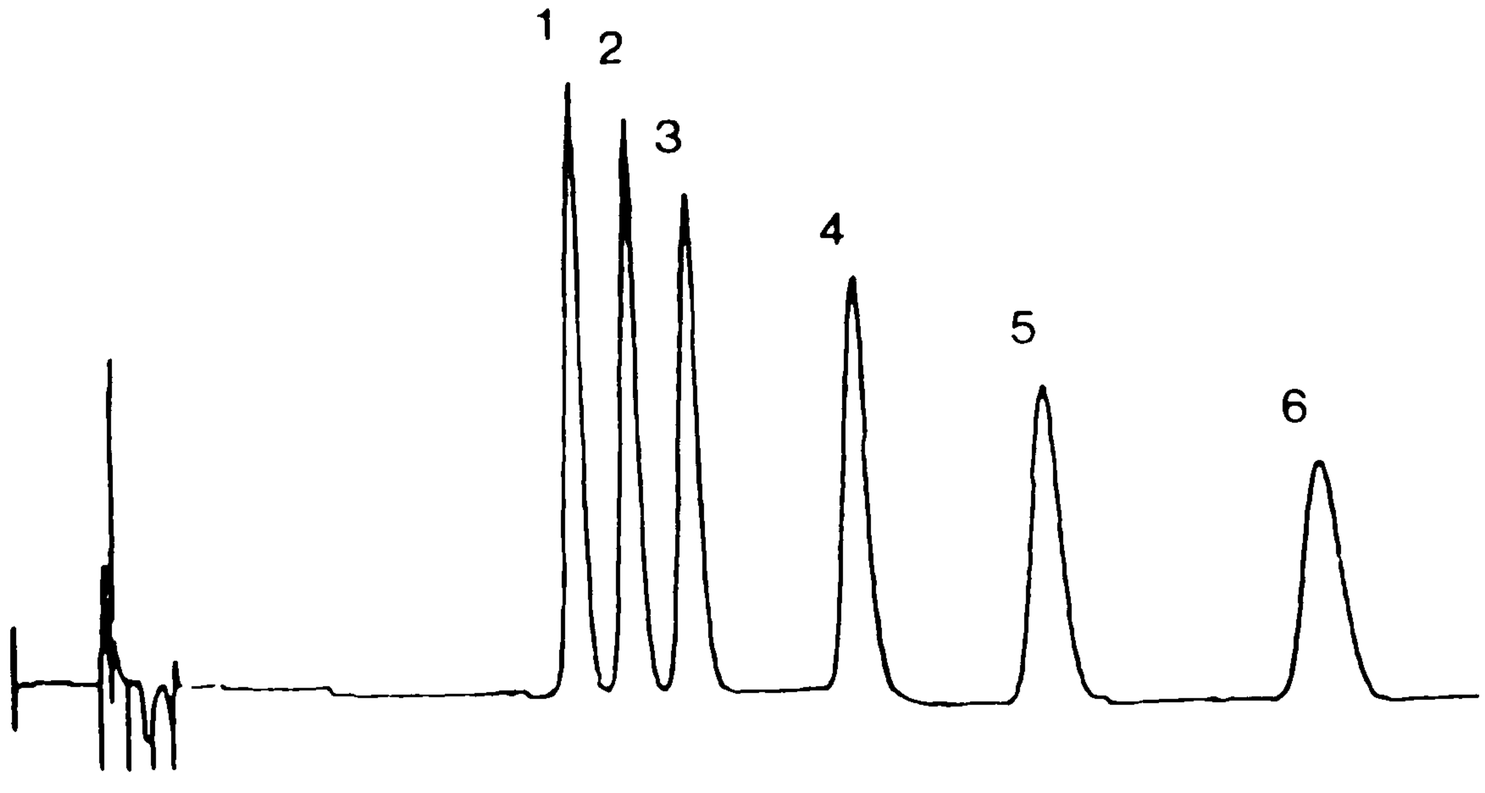


Figure 4.5: Chiral-phase chromatogram of a mixture of carboxyibuprofen and hydroxyibuprofen analysed with a solvent composition of hexane:methanol:ethanol (95:3.5:1.5, v/v) with trifluoroacetic acid (0.05%, v/v). Retention times, peak 1 (2'*S*,2*R*)-carboxyibuprofen: 20.9 min; peak 2 ((*R*)-hydroxyibuprofen): 23.0 min; peak 3 ((2'*R*,2*R*)-carboxyibuprofen): 25.4 min; peak 4 ((*S*)-hydroxyibuprofen): 31.6 min; peak 5 ((2'*R*,2*S*)-carboxyibuprofen): 38.9 min; peak 6((2'*S*,2*S*)-carboxyibuprofen): 49.4 min.

poorer and long retention times would be involved (Figure 4.5). By separately analysing the carboxy and hydroxyibuprofen fractions, better resolution was achieved and the sum of the retention times were the same.

Analysis of standard solutions of racemic carboxyibuprofen yielded peak areas with stereoisomer ratios of 0.25 each. Similarly, enantiomeric ratios for (*R*)- and (*S*)-hydroxyibuprofen enantiomers were 0.5.

4.3.5 Validation of the chiral analytical method

The chiral analysis method was validated by assaying the material in the eluate collected from the normal-phase validation experiments. These validation experiments are essential to determine whether the chiral assay was able to reproduce the expected enantiomeric compositions precisely and accurately throughout the entire analytical procedure and over the range of analyte concentrations. The precision and accuracy values of the calculated enantiomeric ratios for both metabolites are shown in Tables 4.3 and 4.4. The results indicate that there was very little variation from the expected stereochemical compositions over the wide range of concentrations examined, indicating that metabolite concentration does not result in the chiral discrimination using the sequential chromatographic approach. Moreover, even at the lowest concentration used (10 µg/ml), the chiral-phase procedure was sufficiently sensitive to determine the stereochemical composition of the two metabolites in the eluate from the normal-phase system in a reproducible manner. The eluate was generally free of interfering substances that would have otherwise affected the measured enantiomeric compositions. Thus the measurement of enantiomeric ratios using the method described is accurate and precise.

Table 4.3: (a) Within day variation and accuracy and (b) between day variation and accuracy of stereochemical composition determined by the analysis of the eluate from normal-phase analysis of urine samples "spiked" with carboxyibuprofen (n=6).

a)

Concentration (µg/ml)	Isomer	Stereochemical composition (mean ± sd)	Coefficient of Variation (CV%)	Mean Percent difference
10	2'*S*,2*R*	25.05 ± 1.24	4.99	0.21
	2'*R*,2*R*	25.10 ± 1.79	7.13	0.41
	2'*R*,2*S*	25.01 ± 0.51	2.04	0.04
	2'*S*,2*S*	24.84 ± 2.42	9.75	-0.66
80	2'*S*,2*R*	24.97 ± 0.71	2.87	-0.13
	2'*R*,2*R*	25.05 ± 1.52	6.10	0.22
	2'*R*,2*S*	24.99 ± 0.97	3.87	-0.05
	2'*S*,2*S*	24.99 ± 1.17	4.68	-0.03
320	2'*S*,2*R*	25.04 ± 1.34	5.34	0.14
	2'*R*,2*R*	25.01 ± 1.04	4.16	0.02
	2'*R*,2*S*	24.99 ± 1.20	4.81	-0.03
	2'*S*,2*S*	24.97 ± 0.59	2.36	-0.13

Table 4.3 (cont'd)

(b)

Concentration (µg/ml)	Isomer	Stereoisomeric composition (mean ± sd)	Coefficient of Variation (CV%)	Mean Percent difference
10	2'*S*,2*R*	24.99 ± 1.05	4.21	-0.06
	2'*R*,2*R*	25.07 ± 1.75	6.98	0.26
	2'*R*,2*S*	24.99 ± 1.14	4.55	-0.04
	2'*S*,2*S*	24.96 ± 0.47	1.88	-0.17
80	2'*S*,2*R*	24.98 ± 0.35	1.41	-0.07
	2'*R*,2*R*	25.02 ± 1.02	4.06	0.09
	2'*R*,2*S*	24.98 ± 0.33	1.31	-0.08
	2'*S*,2*S*	25.02 ± 0.47	1.87	0.06
320	2'*S*,2*R*	25.06 ± 1.66	6.63	0.22
	2'*R*,2*R*	24.97 ± 0.73	2.94	-0.13
	2'*R*,2*S*	25.02 ± 1.66	6.65	0.06
	2'*S*,2*S*	24.96 ± 0.88	3.52	-0.15

Table 4.4: (a) Within day variation and accuracy and (b) between day variation and accuracy of stereochemical composition determined by the analysis of the eluate from normal-phase analysis of urine samples "spiked" with hydroxyibuprofen (n=6).

(a)

Concentration (µg/ml)	Isomer	Enantiomeric composition (mean ± sd)	Coefficient of Variation (CV%)	Mean Percent difference
10	*R*	50.00 ± 0.36	0.71	0.00
	S	50.00 ± 0.36	0.71	0.00
80	*R*	50.02 ± 1.61	3.21	0.04
	S	49.98 ± 1.60	3.21	-0.04
320	*R*	50.00 ± 0.98	1.96	0.01
	S	50.00 ± 0.98	1.96	0.00

(b)

Concentration (µg/ml)	Isomer	Enantiomeric composition (mean ± sd)	Coefficient of Variation (CV%)	Mean Percent difference
10	*R*	50.05 ± 2.50	5.00	0.09
	S	49.95 ± 2.50	5.01	-0.09
80	*R*	50.05 ± 1.39	2.77	0.10
	S	49.95 ± 1.38	2.77	-0.10
320	*R*	50.12 ± 1.68	3.35	0.25
	S	49.88 ± 1.68	3.37	-0.25

4.4 Summary

In summary, a reliable normal-phase HPLC assay for the determination of carboxyibuprofen and hydroxyibuprofen in urine has been developed and validated. The chiral-phase HPLC method developed was also validated and found to be suitable for the determination of enantiomeric composition of the metabolites in urine. The methodology described above is the first reported method for the determination of the stereochemical composition of the two major metabolites of ibuprofen in urine involving sequential achiral-chiral chromatography and the first method in which the stereoisomers of carboxyibuprofen can be individually quantified following the administration of the racemic drug. The previously reported methods for the analysis of this metabolite have all resulted in co-elution of two of the stereoisomers or their derivatives (Kaiser *et al.*, 1976; Young *et al.*, 1986; Nicoll-Griffith *et al.*, 1988; Baillie *et al.*, 1989) or involved long retention times (run times of greater than 1 hr) (Rudy *et al.*, 1990) and also the chromatographic peaks could not be conclusively identified as to their stereochemical configuration, due to the lack of available reference compounds. The application of the above methodology to the analysis of the metabolites, both free and conjugated with glucuronic acid, in human urine is presented in Chapters 6 and 7.

CHAPTER 5

Determination of Serum Protein Binding of the enantiomers of Ibuprofen

5.1 Introduction

When drugs enter the systemic circulation, they are invariably reversibly bound to plasma proteins. Although human plasma contains over 60 different proteins, it is mainly albumin and α_1-acid glycoprotein which principally act as transport proteins for endogenous and exogenous substances. Generally, albumin tends to be the principal binding protein for acidic drugs such as the NSAIDs, while α_1-acid glycoprotein tends to bind principally to basic drugs like the β-blockers and quinidine (Lin *et al.*, 1987; Wallace and Veerbeck, 1987). The types of molecular interaction involved in drug-plasma protein binding include ionic, hydrogen and dipole interactions, and the binding process is assumed to obey the Law of Mass Action, i.e.

$$\text{drug} + \text{protein} \rightleftharpoons \text{drug - protein complex}$$

Thus the drug-protein complex act as a reservoir that releases more drug as free drug is metabolised or excreted (Mehta, 1989).

As only unbound drug is able to diffuse across biological membranes and interact with receptor sites to elicit a response, the ability to measure drug-plasma protein binding and to understand the effect of this binding on drug disposition is therefore important. Moreover, protein binding has been shown to exhibit stereoselectivity. For example, the *R*-enantiomer of ibuprofen and the *S*- enantiomer of warfarin are more strongly bound to plasma proteins than their respective antipodes (Toon and Trager, 1984; Evans *et al.*, 1989). Thus, the study of the stereoselectivity of plasma protein binding for chiral drugs like ibuprofen, and the factors that affect it, are very relevant in a pharmacokinetic study.

There are two general approaches to the study of plasma protein binding. In the first, only the free drug concentration is determined with respect to the total plasma concentration, to give a measure of the extent of the binding. The techniques involved here include equilibrium dialysis, ultrafiltration,

ultracentrifugation and liquid chromatography, and these approaches yield sufficient information for pharmacokinetic studies. In the second approach, the quantitative relationship between the ligand and the protein is examined. The bound and free drug concentration data are then fitted in various Scatchard type plots to yield information on binding affinity, capacity and the number of binding sites involved. Using these models, the interaction between the ligand and between ligand enantiomers can be analysed. For the purpose of the present study, the first approach was considered to be most relevant.

5.1.1 Methods used for measuring plasma protein binding

A variety of methods can be used to measure plasma protein binding and the relative merits of each have been the subject of several reviews (Rowland, 1980; Lin *et al.*, 1987; Mehta *et al.*, 1989). Equilibrium dialysis is the most popular method by far and is the reference method by which other methods are compared. Plasma samples containing both free and bound drug and drug free isotonic buffer are placed on either side of a semi-permeable membrane. Unbound drug from plasma diffuses through the membrane and at equilibrium, the concentration of the drug in the recipient buffer solution and that unbound in the plasma sample are equal. Analytical determination of the drug concentration on both sides of the membrane will thus enable the free fraction to be calculated. Equilibrium dialysis is relatively inexpensive and yields satisfactory results but factors such as temperature, pH of the recipient buffer, buffer composition and dialysis time may significantly affect results and need to be defined. The major disadvantage of the approach is that of volume shifts during dialysis caused by diffusion of water from the buffer compartment to the "protein" side of the dialysis cell. When volume shifts are substantial and unbound concentrations are low, a correction factor must be applied (Lockwood and Wagner, 1983; Lima *et al.*, 1983). This problem is aggravated in cases where protein binding is concentration dependent and results in such instances need to be interpreted with caution (Huang, 1983).

Ultrafiltration is another popular technique, which with the recent availability of specially designed centrifugal devices is being increasingly used. A typical device consists of a sample reservoir and filter unit containing a low absorption membrane with a molecular weight cut-off of about 30,000 Daltons (D). The plasma sample containing drug is placed in the sample reservoir and the unit centrifuged at the required g-force for an appropriate time under conditions of controlled temperature. The protein free ultrafiltrate collected beyond the membrane is analysed to assess the free drug concentration . The method is fast, easy and reliable, although the filter devices are relatively expensive. The biggest setback of this method is that of drug-binding to the membrane, which should be investigated before application of the method. This is usually carried out using radiolabelled drug (Mehta, 1989).

Ultracentrifugation, which involves separating free drug from the drug-protein complex by high speed centrifugation is theoretically the ideal method as it neither disrupts equilibrium or results in sample dilution. However, it requires an expensive centrifuge, large volumes of sample and long centrifugation times. It is also susceptible to variations caused by drug binding to the tubes and low density lipoproteins, which rise to the top of the tube during centrifugation (Rowland, 1980).

An alternative technique involves gel-filtration or size exclusion chromatography, the unbound drug being trapped in the pores of the gel of the stationary phase, while large protein molecules pass through the column relatively unimpeded. The eluate containing the free drug is collected and then analysed. The method is simple but problems arise if on-column dissociation of the drug-protein complex occurs. Also, relatively large samples are required and it is generally more useful for *in-vitro* binding studies (Rowland, 1980).

Other methods such as differential spectrophotometry, circular dichroism, affinity chromatography and measurements of altered physicochemical properties of

drug-protein solutions are less commonly used methods, which are employed for an examination of the mechanistic aspects of drug-protein binding (Lin *et al.*, 1987).

5.1.2 Analytical requirements for the determination of unbound drug

For drugs which are highly protein bound, e.g warfarin, phenytoin and the 2-APAs, free drug assays need to be very sensitive as extremely low concentrations are encountered. Often this analytical requirement necessitates the use of radiolabelled drugs of high specific activity (Rowland, 1980). However, the radiolabelled material must be of high purity, both chemical and radiochemical, otherwise misleading values will be obtained (Bjornsson *et al*, 1981). In the case of ibuprofen, previous studies have indicated that protein binding is greater than 99% (Gallo *et al*, 1986) and that the binding is stereoselective for the *R*-enantiomer (Evans *et al.*, 1989). Thus, with total drug enantiomer concentrations expected between 1 - 20 µg/ml, free concentrations in the low nanogram per ml range for both enantiomers of ibuprofen are expected and the analytical method used requires a minimal quantifiable limit in this range.

In order to study the enantioselective disposition of ibuprofen in human volunteers, an HPLC method with adequate sensitivity for the direct analysis of the free concentrations of ibuprofen enantiomers was developed and is described below. This method is a modification of that developed for serum and urine samples described in Chapter 2 and determines directly free drug concentrations without resorting to radiolabelled compounds. During the course of the investigations described in this chapter, a sample of radiolabelled [^{14}C]-ibuprofen became available and additional validation experiments were carried out by comparison of the results obtained using the developed assay with a previously published radiochemical procedure (Evans *et al.*, 1989). Equilibrium dialysis was preferred over the other methods of determining free concentrations because of the advantages discussed earlier, i.e. it is a relatively cheap to perform and does not require specialised instrumentation.

5.2 Experimental

5.2.1 Chemicals and materials

Sodium dihydrogen phosphate, disodium hydrogen phosphate and sodium chloride (Analar grade) were obtained from BDH (Poole, Dorset). Dialysis membranes (Spectrapor 2) were obtained from Pierce and Warriner (Chester, U.K.). Protein Assay reagents based on the Coomasie blue method were obtained from Bio-Rad (Herts, U.K.). Stainless steel Resolve C_{18} column (150 x 3.9mm, 5 µm) and Radialpak Resolve C_{18} HPLC cartridges (100 x 6 mm, 5 µm) were obtained from Anachem (Luton, Beds., U.K.). Radiolabelled [^{14}C]-ibuprofen with specific activity of 21.6 mCi/g was donated by Knoll Pharmaceuticals (Nottingham). The liquid scintillation cocktail used was Quickscint flow 302 (Zinsser Analytical, Berkshire, U.K.). Liquid scintillation determinations were carried out using an LKB Wallac 1209 Rackbeta liquid scintillation spectrometer (Milton Keynes, U.K.) Dialysis cells made of perspex, of internal volume of 1.25 ml and the dimensions of which are given in Appendix 7 were made in the Pharmacy Department workshop of King's College London. Other reagents and materials were similar to those listed in section 2.2.1.

5.2.2 Purification of [^{14}C]-ibuprofen.

The radiolabelled ibuprofen was purified by HPLC prior to use. [^{14}C]-Ibuprofen (3 mg) was dissolved in acetonitrile (1 ml) and 50 µl aliquots were injected onto a reversed-phase Resolve C_{18} column (150 x 4.6 mm, 5 µm) with a mobile phase of acetonitrile:water (40:60, v/v) with the pH adjusted to 3.5 with hydrochloric acid (1.0 M) at a flow rate of 1 ml/min. Detection was by UV, set at 220 nm. The eluate containing ibuprofen was collected from the detector outlet into a 100 ml pear shaped flask. The combined eluate (from 20 injections) was then pooled and the solvent evaporated to half its volume under nitrogen gas at 40^{0}C. Hexane:isopropanol (9:1, v/v; 25 ml) was added as extraction solvent and the

mixture well shaken. The solvent mixture was allowed to separate and the supernatant organic layer was transferred into a 50 ml pear shaped flask and evaporated to dryness under nitrogen gas. The chemical purity of the product was subsequently determined by redissolving a portion of the residue in mobile phase and reinjecting it onto the HPLC system. Purification of the radiolabelled drug was performed immediately prior to use in subsequent equilibrium dialysis experiments.

5.2.3 Equilibrium dialysis

Spectrapor 2 dialysis membranes were cut into 3.5 x 3.5 cm squares and soaked overnight in phosphate buffered saline. Prior to use, the membranes were carefully placed between the two halves of the dialysis cells and the cells held in place using the screws provided. Serum samples (3 x 1 ml) were carefully transferred into three adjacent dialysis cells. Equal volumes of phosphate buffered saline were transferred into the opposite sides of the membranes. The ports of the cells were then sealed using plastic stoppers and the cells placed in a shaking water bath set at 37°C and a shaking frequency of 30 strokes per minute. The cells were then allowed to equilibrate for a preset equilibrium time of 8 hours (see section 5.2.5).

5.2.4 Determination of protein concentration

The protein concentration of the serum samples before and after dialysis were determined using the Coomasie blue procedure (Bio-Rad Protein Assay kit) in order to correct for possible volume shifts during the dialysis experiments. Serum samples (25 µl) were diluted 1:40 (v/v) with phosphate buffered saline. Protein standard solutions consisting of 0, 0.0875, 0.175, 0.35, 0.7 and 1.4 mg/ml bovine serum albumin in phosphate buffered saline. The standard solutions or diluted serum samples (100 µl) were pipetted into 10 ml dry test-tubes and 5.0 ml of Coomasie dye reagent (5 ml) was added to each tube. After gentle mixing, the

solutions were left to react for five minutes and the absorbance at 595 nm was then determined. Standard calibration curves were constructed using the protein standard solutions and the protein concentrations of the serum samples were determined against this calibration curve. The ratio of the protein concentrations (F) after and before dialysis were determined for each serum sample.

5.2.5 Determination of time to achieve equilibrium

To determine the time required for drug concentrations to reach equilibrium across the membrane, an experiment with radiolabelled drug was carried out. Radiolabelled ibuprofen was dissolved in acetonitrile added to phosphate buffered saline to obtain a concentration of 15 µg/ml of racemic drug. The solution was then dialysed against drug free phosphate buffered saline. Aliquots (25 µl) were taken from both sides of the dialysis cells were removed at regular (hourly intervals from 0 - 5 hours, then 2 hourly from 5 to 9 hours) and the radioactivity determined. The radioactivity (expressed in dpm) of the donor and recipient samples were plotted against time of sampling to determine the time to achieve equilibrium. This procedure was repeated twice (n = 3).

5.2.6 Analysis of ibuprofen enantiomers in equilibrium dialysate

(a) Column packing procedure

A semi-microbore (2.1mm i.d.) column was packed in-house in order to achieve an increase in mass detectability of the HPLC procedure. An empty HPLC column was constructed out of an empty stainless steel tubing (150 x 2.1 mm), one end of which was attached a Swagelok compression fitting with a 2 µm stainless steel frit. The empty column was attached to HPLC column packer set in an upward packing configuration. Loose Resolve C_{18} media was obtained by emptying a Resolve C_{18} radialpak cartridge. A 1.5g portion was transferred into a 100 ml

conical flask. The powder was suspended in 10 ml of methanol and sonicated for 5 minutes. The suspension was then quickly transferred into the reservoir of the HPLC column packer and the solvent lines were quickly attached. The column was packed at a pressure of 400 bar using isopropanol as solvent. After about 30 ml of solvent had eluted, the column was then switched to a downward position and packing continued until another 30 ml of solvent had eluted. The packing solvent was then changed to methanol and packing continued until another 30 ml of solvent had eluted. The column was removed after residual pressure had dissipated and the second Swagelok end-fitting attached. The column was then attached to the HPLC and equilibrated with acetonitrile at a flow rate of 0.2 ml/min overnight.

(b) Extraction procedure

To the dialysate buffer (2.5 ml: the contents of three cells combined) was added flurbiprofen internal standard solution (5 µg /ml in acetonitrile, 25 µl), followed by 0.5 ml HCl (1.0M) and 2 ml of phosphate buffer (1.0M, pH 3.8). and the whole extracted with hexane:isopropanol (9:1, v/v, 5 ml) as described previously (section 2.2.4 (c)).

(c) Derivatization

To the dry residues were added 30 µg each of CDI, HOBT and (*R*)-NEA (30µl of a 1 mg/ml solution in dichloromethane). The tubes were then tightly capped and the derivatization carried out as described in section 2.2.5 (b). The final residues were dissolved in 50 µl of HPLC mobile phase and 30 µl was injected into the HPLC.

(d) Chromatography

The semi-microbore column packed using the procedure described in 5.2.6(a) was used. The mobile phase was 50:50 (v/v) mixture of phosphate buffer (0.01M, pH 3.5):acetonitrile at a flow rate of 0.6 ml/min. Detection was by

fluorescence with excitation and emission wavelengths set at 290 and 330 nm respectively. Calibration curves were constructed from analysis of dialysate solutions "spiked" with racemic ibuprofen to obtain concentrations of 5, 10, 20, 40, 80 and 160 ng/ml of each enantiomer. The concentration of each enantiomer in the dialysate samples were determined by comparing their respective peak area ratios (ibuprofen enantiomer: (*S*)-flurbiprofen derivatives) to the calibration curve prepared and the unbound fraction (f_u') determined by comparing the enantiomer concentrations with the corresponding total (free and bound) enantiomer concentration of the samples determined using the serum assay procedure (Chapter 2). The unbound fractions were then corrected for volume shifts using the following equation (eqn 5.1):

$$f_u = f_u' \times F/(f_u' \times F + 1 - f_u') \qquad \text{(eqn 5.1)}$$

where F is the ratio of the serum protein concentration after and before dialysis, and f_u is the unbound concentration after volume shift correction (Huang, 1983).

(e) Validation of the HPLC procedure

A stock solution of 1 mg/10 ml of (*R,S*)-ibuprofen in acetonitrile was prepared and aliquots of 10, 20, 40, 80 , 160 and 320 µl were pipetted into six 100 ml volumetric flasks. The solvent was evaporated gently under nitrogen and drug free dialysate buffer was added q.s. 100 ml to give concentrations of 5, 10, 20, 40 ,80 and 160 ng/ml of each enantiomer. On each day of analysis, 2.5 ml of these solutions were pipetted out into separated tubes and analysed. Calibration curves were constructed based on these data. The accuracy and within day variation of the assay was determined by analysing the calibration samples of 5, 40 and 160 ng/ml concentrations repeatedly (n=6). The recovery of the extraction procedure was determined as before (section 2.2.5(d)), by comparing peak areas obtained with direct injections of standard solutions of equivalent concentrations of the drug

Between day variation was determined by analysing the same solutions over six separate days.

5.2.7 Validation of HPLC procedure against the radiolabelled method

The HPLC method described above was compared with a published procedure using radiolabelled ibuprofen (Evans *et al.*, 1989). Serum samples "spiked" with 5, 10 and 20 µg/ml of each ibuprofen enantiomer were used for this validation experiment. Aliquots of serum samples (3 x 1 ml) were transferred into three dialysis cells. Into each cell was also added 20 µl of [^{14}C]-ibuprofen (0.35 mg/ml) dissolved in human serum albumin solution (4 % w/v in water). On the opposite side of the cells (recipient) were placed equal volumes of phosphate buffered saline. The cells were then incubated as described above (section 5.2.3). After incubation, a 400 µl aliquot of serum and buffer were removed from the cells and placed in separate scintillation vials along with 12.5 ml scintillation cocktail. The radioactivity was then determined using a liquid scintillation spectrometer. The ratio of the radioactivity between the donor and recipient sides of the cells (expressed in dpm) was then calculated.

A 2.5 ml portion of the recipient buffer was extracted, derivatized and analysed as described in section 5.2.5. The eluate containing the radiolabelled diastereoisomeric amides were collected from the detector outlet and transferred into separate scintillation vials and the radioactivity determined as before (dpm *R* -B and dpm *S* -B). The procedure was repeated using 200 µl of equilibrated serum (dpm *R* -P and dpm *S* -P). The unbound fraction of (*R*)-ibuprofen was determined as

$$f_{U,R} = \frac{\text{dpm B}}{\text{dpm P}} \times \frac{\text{dpm } R\text{ - B}}{\text{dpm } R\text{ - B} + \text{dpm } S\text{ - B}} \Big/ \frac{\text{dpm } R\text{ - P}}{\text{dpm } R\text{ - P} + \text{dpm } S\text{ - P}} \qquad \text{(eqn 5.2)}$$

and that of (*S*)-ibuprofen as:

$$f_{u,s} = \frac{\text{dpm B}}{\text{dpm P}} \times \frac{\text{dpm } S\text{ - B}}{\text{dpm } R\text{ - B} + \text{dpm } S\text{ - B}} \Big/ \frac{\text{dpm } S\text{ - P}}{\text{dpm } R\text{ - P} + \text{dpm } S\text{ - P}} \qquad \text{(eqn 5.3)}$$

Free enantiomer concentrations were calculated by multiplying the respective unbound fraction values by the total (bound and unbound) enantiomer concentration. The results obtained for the free concentrations and unbound fractions for the individual enantiomers were compared to those obtained for the same serum samples analysed directly by the HPLC procedure.

5.3 Results and Discussion

5.3.1 Purification of [^{14}C]-ibuprofen

Based on peak areas, the purity of the radiolabelled ibuprofen before chromatographic purification was about 96% and after purification it was estimated to be 99%, based on peak areas. The purification process was carried out prior to use in order to ensure radiochemical purity. A preliminary dialysis experiment was carried out to compare the extent of protein binding in spiked serum samples (20 µg/ml) of the radiolabelled drug before and after HPLC purification. Based on dpm values of the serum and dialysate buffer samples, the unbound fraction for total ibuprofen was estimated to be 1.8% before purification and 0.5% after purification. This is consistent with previously reported findings (Evans *et al.*, 1989). Thus, it is obvious that purification of the radiolabelled material is essential to obtain an accurate estimate of the extent of protein binding and that variations in results from previously published findings may depend on the how rigorously this purification process is carried out.

5.3.2 Determination of time to achieve equilibrium

A plot of radioactivity versus time for both the donor and recipient compartments of the dialysis cells is shown in Figure 5.1. Equilibrium was achieved after about 8 hours and as such this was used as the incubation time for subsequent dialysis experiments.

5.3.3 HPLC analysis of ibuprofen enantiomers in equilibrium dialysate

The assay procedures used to determine the free enantiomer concentrations are basically similar to those developed for drug enantiomer analysis in serum and urine (Chapter 2). Much larger volumes of samples and dialysate buffer were required compared to those used previously in order to increase assay sensitivity and as a result of this, the volumes of extraction buffer and acid had to be proportionately increased in order in ensure sufficient buffering capacity. In addition 30 µg each of the derivatizing reagents were used instead of the 100 µg normally used in the analysis of serum and urine samples. This proportionate decrease was necessary in order to control the background noise in the chromatographic analysis, which was critical as only low nanogram amounts were injected on-column. The validation experiments in the serum analysis showed reliable quantitation down to 100 ng/ml (see Chapter 2). Further efforts to reduce the limit of quantitation revealed a lower limit of 25 ng on column. To achieve a 2 to 3 fold increase in assay sensitivity, a smaller bore column was thought to be the best approach, as it would increase mass detectability and assay sensitivity without requiring major changes in the analytical procedure. However, smaller bore columns with the same C_{18} media are not commercially available, and neither is the loose media. Thus, the C_{18} media of the required chemistry was obtained by emptying a 100 x 6 mm Radialpak cartridge, which yielded 2.5g of material. The packed C_{18} column showed the same selectivity as the C_{18} column used earlier and baseline resolution was achieved between the peaks of interest. Due to lower peak dispersion, sensitivity was increased and an on-column injection of 5 ng of the

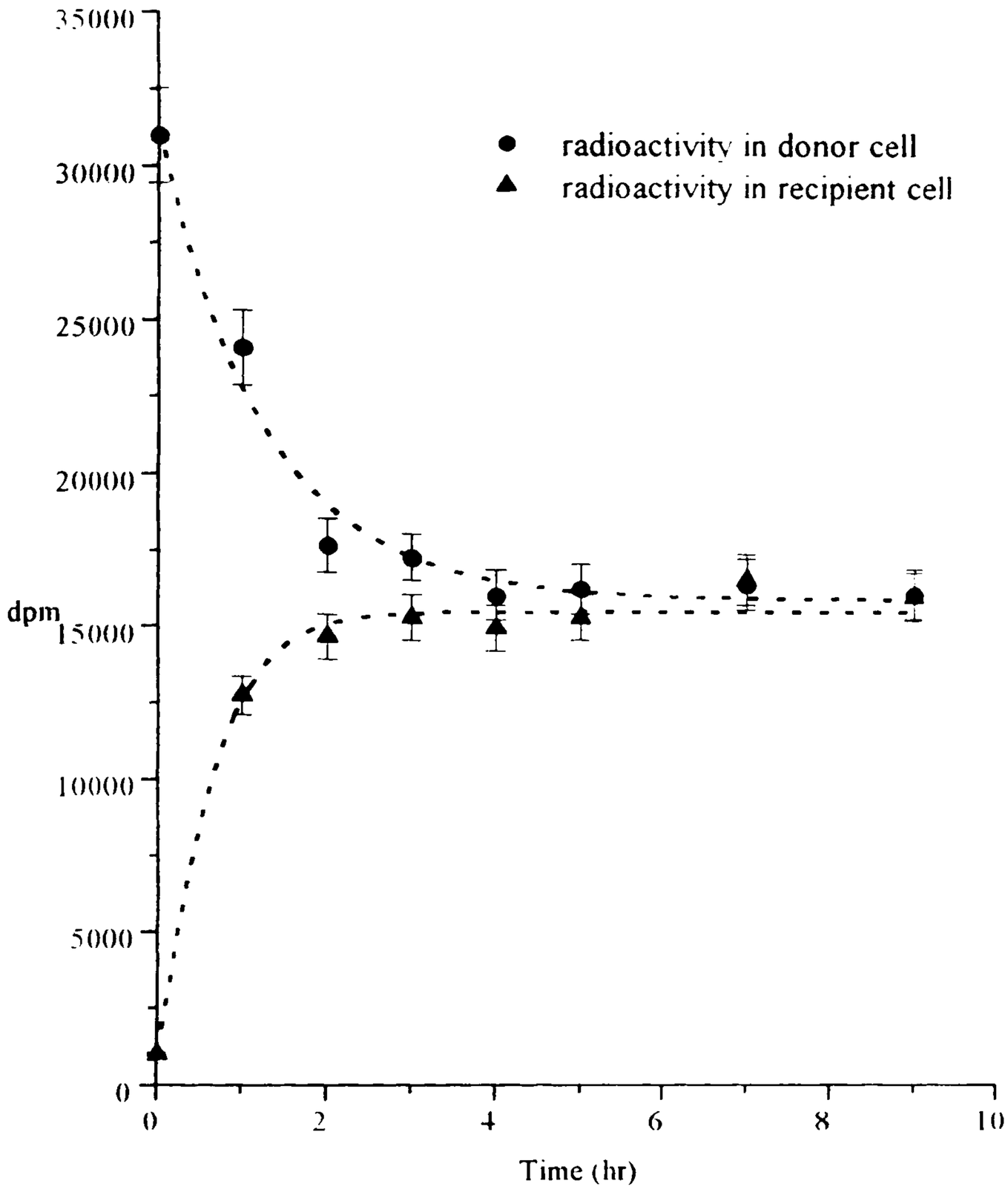


Figure 5.1: Plot of radioactivity (dpm) versus time of equilibrium of ^{14}C]-ibuprofen in the donor and recipient cells filled with phosphate buffered saline (mean ± sd, n = 3).

diastereomeric amides yielded sufficiently large peaks to enable reliable quantitation (see below). Connection tubing of the conventional HPLC were also reduced in order to reduce peak dispersion further. Chromatograms of ibuprofen derivative standards, blank dialysate and dialysate of a volunteer's serum sample are shown in Figure 5.2. Baseline resolution was achieved and no interfering peaks were evident. Peak symmetry was not as good as with the regular columns although it did not affect the resolution. This was probably due to relatively low packing pressure of 400 bar that was employed in the packing procedure, which was the maximum tolerable limit of the column packer.

5.3.4 Validation of the HPLC assay procedure

The accuracy, extraction recovery and within and between day variability of the assay procedure is shown in Table 5.1 and typical calibration curves are presented in Figure 5.3. In general the variation is much larger than that seen for the urine and serum assay procedure. This is not unexpected as much lower concentrations are involved. However, even at the lowest calibration point of 5 ng/ml the within day variation is about 13 % and the day to day variation was about 16% , which is still within acceptable limits, considering the low concentrations involved. This is especially true when applied in protein binding studies where much larger experimental variations are usually encountered (Huang, 1983). Thus, the minimum quantifiable limit of the method is 5 ng/ml of each enantiomer, using 2.5 ml of protein dialysate. This low limit of quantitation is therefore sufficient for the determination of plasma protein binding in serum samples with a total (bound and unbound) enantiomer concentration of at least 5 µg/ml, and can thus be used in human pharmacokinetic studies.

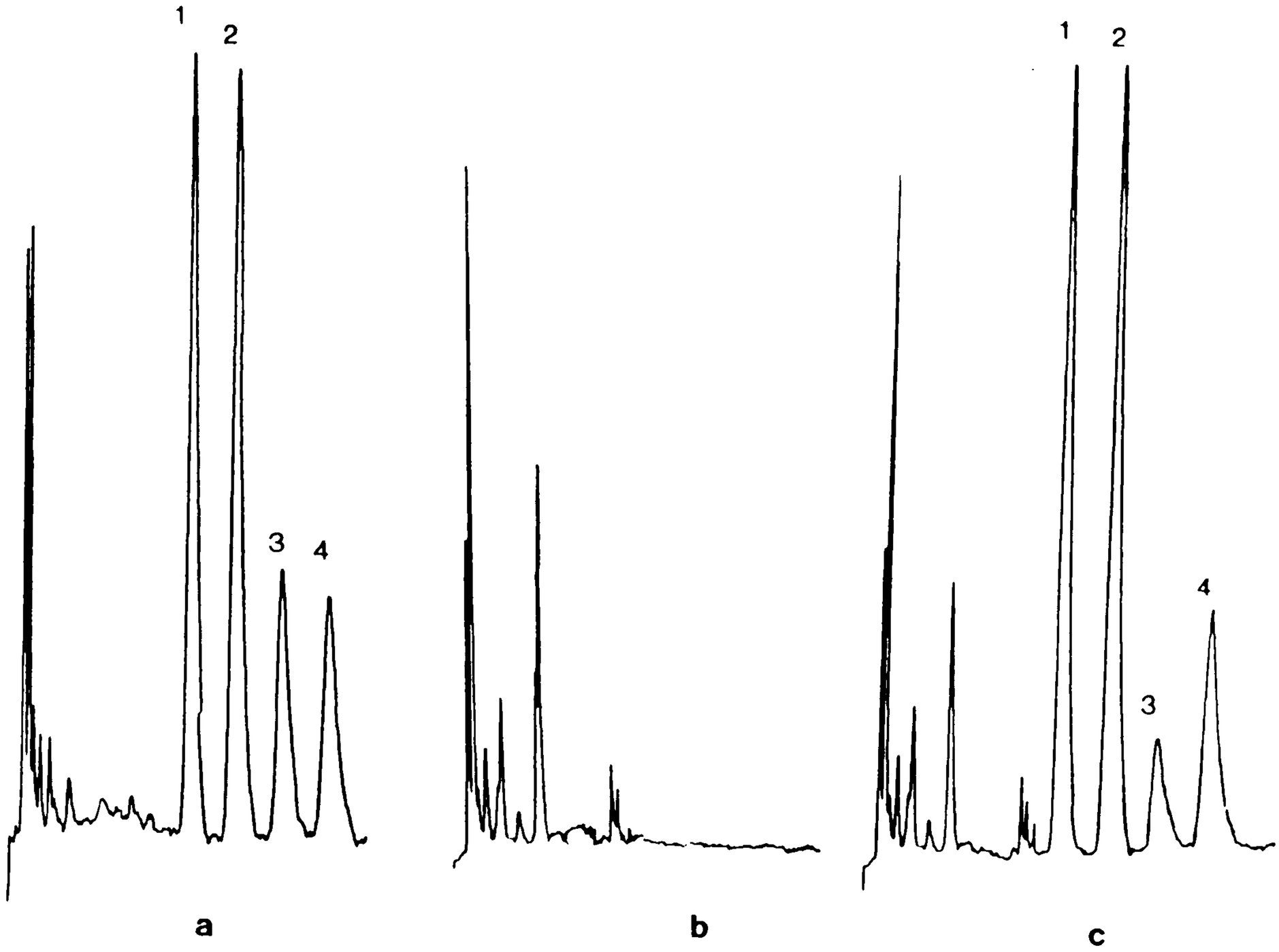


Figure 5.2 Chromatograms of a) standard solutions of racemic ibuprofen 160 ng/ml of each enantiomer, b) dialysate from drug free serum sample, c) dialysate from a serum sample of a volunteer 2.0 hr after oral administration of 400 mg of the racemic drug (see Figure 2.2 for peak identity).

Table 5.1: **(a) Within day variation, accuracy and extraction recoveries of ibuprofen enantiomers in "spiked" protein dialysate samples (n=6) and (b) between day variation and accuracy of ibuprofen enantiomers in "spiked" protein dialysate samples (n=6).**

(a)

Concentration (ng/ml)	Enantiomer	Concentration Determined (ng/ml)	Coefficient of Variation (CV%)	Mean Percent Difference	Recovery (%)
5	*R*	4.4 ± 0.6	14.3	-12.0	90.1 ± 12.9
	S	4.6 ± 0.6	12.8	-8.0	92.2 ± 11.8
40	*R*	38.5 ± 1.6	4.2	-3.8	94.4 ± 3.6
	S	38.2 ± 2.5	6.6	-4.5	93.7 ± 6.2
160	*R*	162.1 ± 7.0	4.3	1.3	92.3 ± 3.4
	S	158.5 ± 5.4	3.4	-0.9	91.6 ± 3.1

b)

Concentration (ng/ml)	Enantiomer	Concentration Determined (ng/ml)	Coefficient of Variation (CV%)	Mean Percent Difference
5	*R*	4.5 ± 0.7	15.1	-10.0
	S	4.6 ± 0.7	16.6	-8.0
40	*R*	36.5 ± 3.9	10.8	-8.8
	S	37.2 ± 3.7	9.9	-7.0
160	*R*	165.7 ± 6.6	4.0	3.6
	S	154.2 ± 5.7	3.7	-3.6

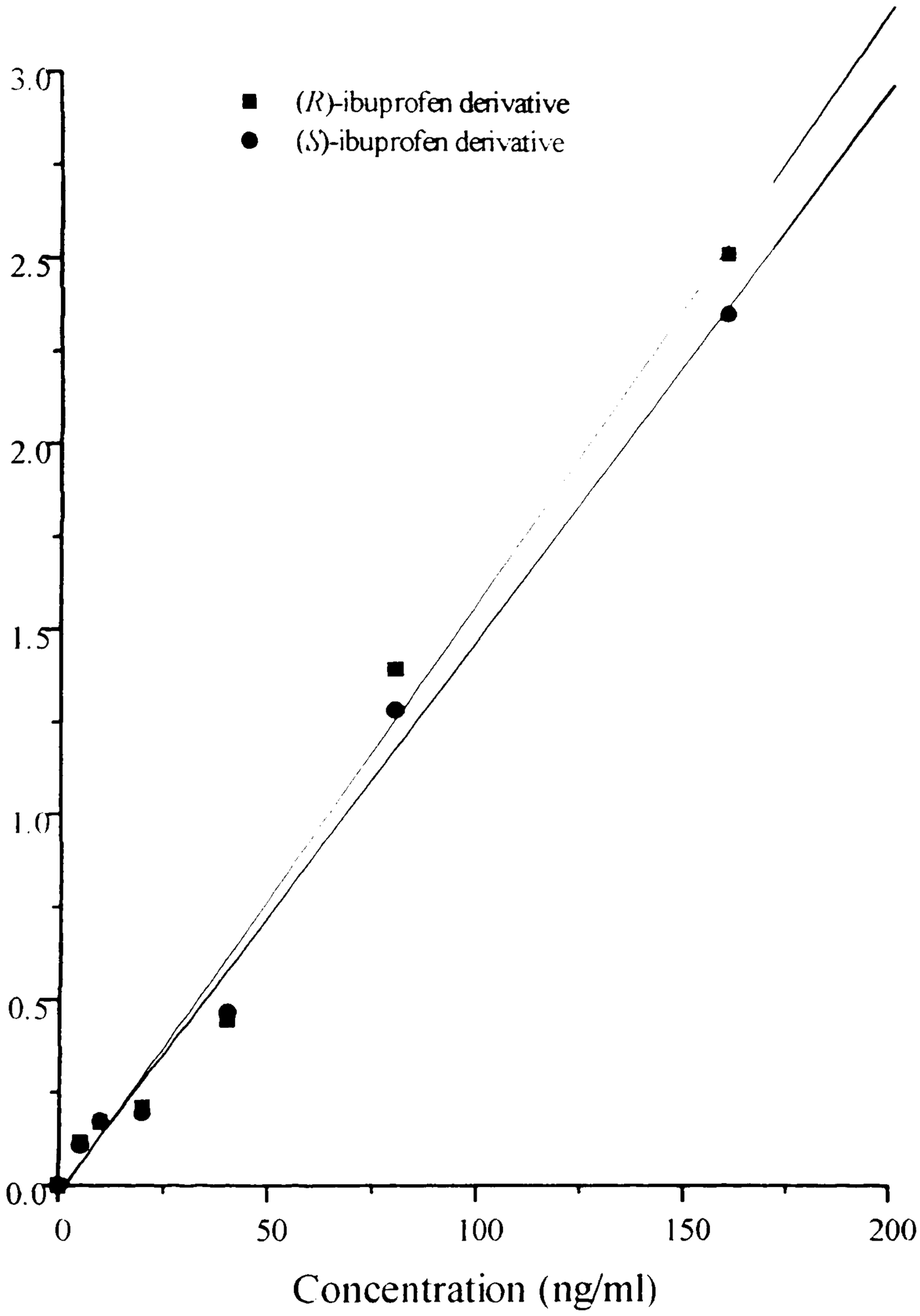
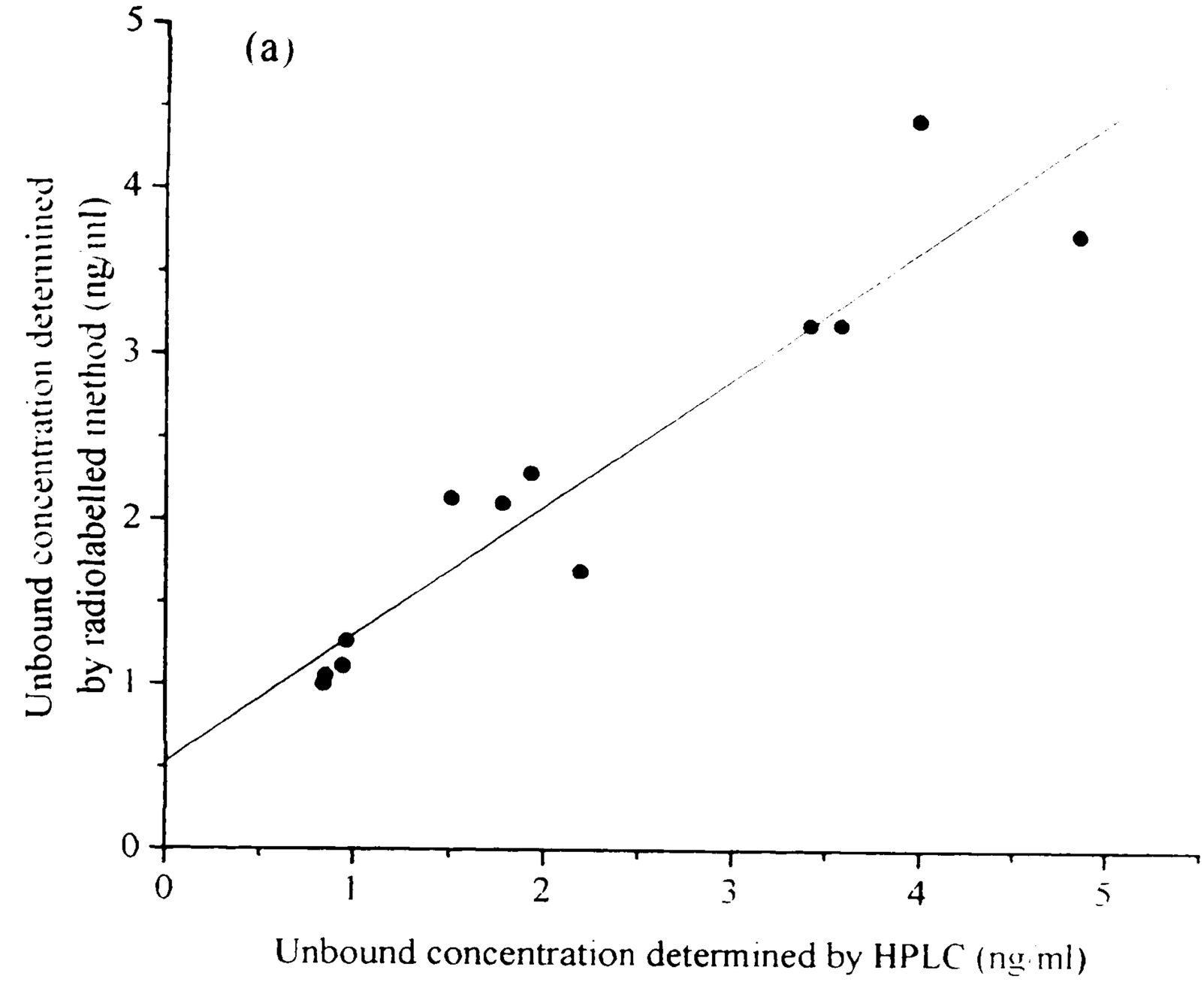


Figure 5.3: Typical calibration curves prepared for the quantitation of ibuprofen enantiomers as their (*R*)-1-(naphthen-1-yl)ethylamides following extraction of the drug from dialysate buffer after equilibrium dialysis.

5.3.5 Validation of HPLC procedure against radiolabelled method

In view of the difficulties involved in plasma protein determination experiments and the low free drug concentrations involved it was thought necessary to validate the chromatographic procedure developed against the previously published procedure involving radiolabelled ibuprofen (Evans *et al.*, 1989). The procedure used in the radiolabelled method was adopted without major modifications. The free drug concentrations determined for the "spiked" serum samples using both the chromatographic and radiolabelled ibuprofen method are presented in Figure 5.4. A linear relationship is evident from the two plots ($r = 0.94$ and $r = 0.90$ for (*R*)- and (*S*)-ibuprofen respectively) indicating that the two sets of values were linearly related. The gradient of the regressed line ($m = 0.9$ and $m = 1.1$ for (*R*)- and (*S*)-ibuprofen respectively) also show that the results obtained from both methods were within 10% of each other, implying a high degree of correlation. The unbound fraction of each enantiomer for the serum samples were also calculated and the results tabulated in Table 5.2. The mean values were not statistically different after being subjected to a Student's t-test for paired samples ($p > 0.05$). Thus, it is apparent that the two methods of determination of free drug concentration and unbound fractions yield comparable results and the chromatographic method can be used with confidence in the determination of the extent of plasma protein binding of ibuprofen enantiomers. The chromatographic method has the added advantages of being free from the hazards of handling and disposal of radiolabelled substances as well as being simpler and less tedious to perform.

Volume shifts in equilibrium dialysis occurs as a result of the movement of water from the recipient to the donor cells causing the dilution of the binding protein concentration and if uncorrected, will result in an overestimation of the unbound fraction. Correction for volume shift is especially important if the unbound fraction of the drug is small (Huang, 1983). The volume shifts in the validation experiments were typically between 7-10 % and the unbound fractions determined in the subsequent pharmacokinetics studies (Chapters 6 and 7) were



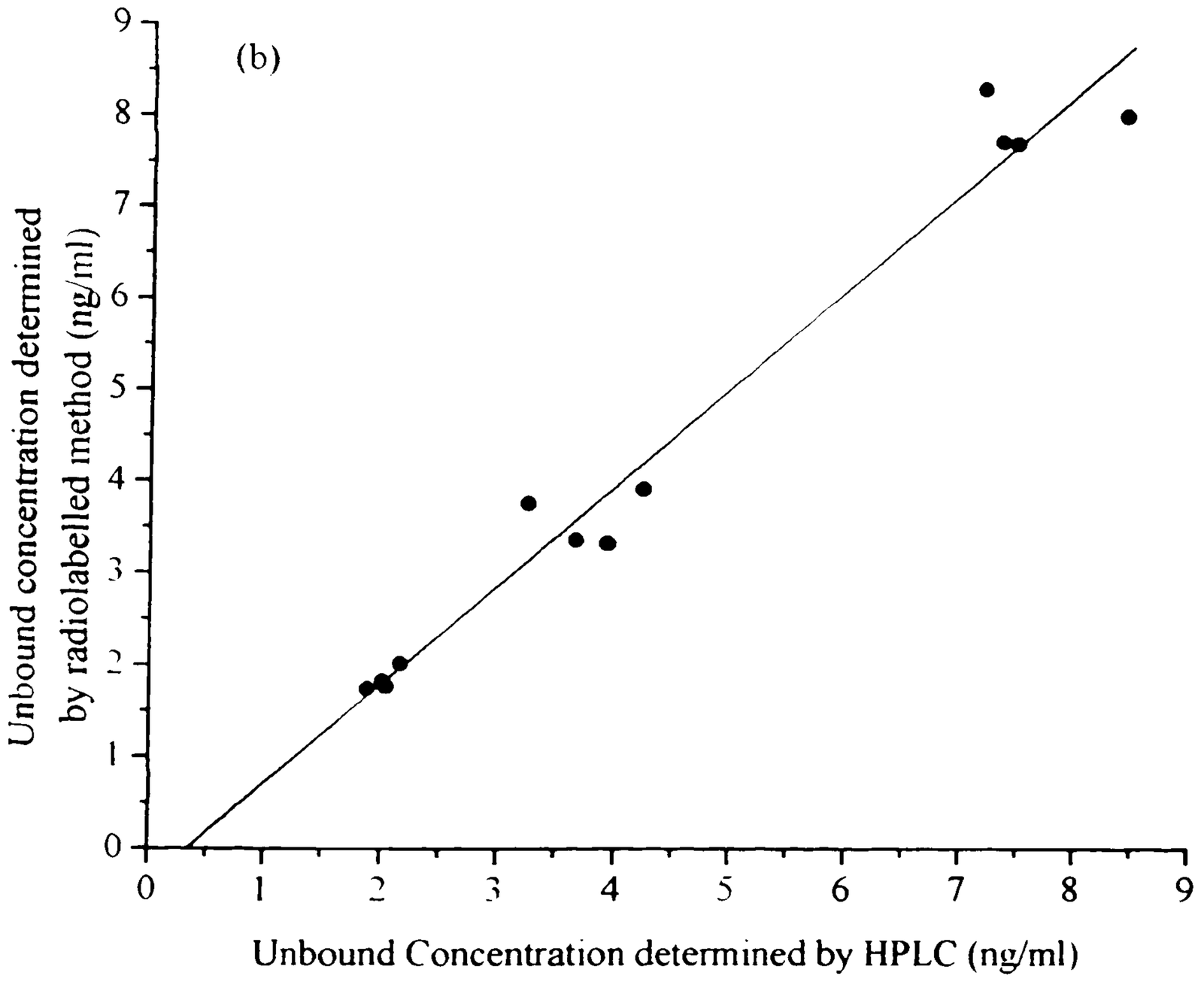


Figure 5.4: **Plot of unbound concentrations of a) (*R*)-ibuprofen and b) (*S*)-ibuprofen determined using HPLC versus that using the radiolabelled ibuprofen method for serum samples spiked with racemic ibuprofen.**

Table 5.2: Unbound fraction of (*R*)- and (*S*)-ibuprofen in "spiked" serum samples determined by both the HPLC and radiolabelled methods using [^{14}C]-ibuprofen.

Enantiomer Concentration	Unbound Fraction of (*R*)-ibuprofen (%)		Unbound Fraction of (*S*)-ibuprofen (%)	
	HPLC	[^{14}C]-ibuprofen	HPLC	[^{14}C]-ibuprofen
5 µg/ml	0.31	0.29	0.68	0.70
	0.44	0.34	0.66	0.76
	0.36	0.40	0.77	0.73
	0.32	0.29	0.67	0.70
10 µg/ml	0.36	0.28	0.54	0.63
	0.25	0.36	0.66	0.55
	0.32	0.38	0.71	0.65
	0.30	0.35	0.61	0.56
20 µg/ml	0.28	0.36	0.59	0.50
	0.27	0.32	0.62	0.57
	0.24	0.28	0.54	0.50
	0.24	0.30	0.58	0.52
mean	0.31	0.33	0.64	0.61
s.d.	0.06	0.04	0.07	0.09
p-value	n.s.		n.s.	

Note: Comparison of the mean unbound fractions determined by the two methods were tested for significance using a Student's t-test for paired samples (n.s. = $p > 0.05$).

routinely corrected for volume shifts based on protein concentration determinations before and after dialysis for every serum sample.

5.4 Summary

In summary, a reliable and accurate method of determination of free drug concentration in serum samples was developed and validated against a previously published procedure using radiolabelled ibuprofen. The method is relatively simple and easy to perform. The extent of plasma protein binding was determined using equilibrium dialysis and volume shifts were typically between 7-10 % during the validation experiments. Equilibrium dialysis was easy to perform and did not require the use of specialised apparatus. This method of determination was applied to serum samples obtained from human pharmacokinetic studies following the oral administration of 400 mg racemic ibuprofen, as described in Chapters 6 and 7.

CHAPTER 6

Enantioselective Pharmacokinetics of Ibuprofen in Young Volunteers.

6.1 Introduction

Human pharmacokinetic studies of (*R,S*)-ibuprofen are complicated by three factors. Firstly, the drug is mainly used as a racemic mixture and the unidirectional chiral inversion from the inactive *R*- to the active *S*-enantiomer represents a form of metabolic activation, the extent and variability of which need to be studied in order to obtain an estimate of the dose of the active drug that is presented to the body. This will allow subsequent determination of clearance via other non-inversion processes. Variability in chiral inversion will also affect other stereoselective transformations and may well result in marked differences in enantiomeric disposition (Caldwell *et al.*, 1988).

Secondly, ibuprofen is extensively bound to plasma proteins (Gallo *et al.*, 1986; Lin *et al.*, 1987) with unbound fractions of typically less than 0.01. Plasma protein binding has also been shown to be enantioselective in *in-vitro* and *in-vivo* studies, with the *S*- enantiomer having a significantly higher unbound fraction (Evans *et al.*, 1989; 1990). Like others compounds in the "profen" group, ibuprofen is cleared almost exclusively by hepatic metabolism (Williams *et al.*, 1993) and the clearance (CL) of such drugs is given by:

$$CL = f_u \, CL_u \qquad \text{(eqn 6.1)}$$

where f_u is the unbound fraction and CL_u is the unbound or intrinsic clearance of the drug (Rowland and Tozer, 1989). Therefore , stereoselective differences in total clearance may be due either to differences in unbound clearance or in protein binding. Thus, pharmacokinetic studies based on total (bound and unbound) determinations alone will not yield information on the enantioselectivity of the underlying dispositional processes. Due to the large extent of protein binding, very low unbound concentrations of each enantiomer are encountered and this represents a severe analytical challenge. As a result, pharmacokinetic studies of ibuprofen enantiomers based on unbound drug concentrations are rare.

Thirdly, in addition to chiral inversion, ibuprofen undergoes functional oxidative and conjugative metabolism. The excretion of ibuprofen as the acyl glucuronide accounts for about 10% of the dose. Although this is quantitatively a relatively unimportant pathway, *in-vitro* experiments reveal the glucuronidation to be stereoselective (El Mouehli *et al.*, 1987). The major metabolic pathway is oxidation, to yield two principal metabolites, namely hydroxyibuprofen and carboxyibuprofen (Figure 6.1). In the case of carboxyibuprofen, a second chiral centre is introduced into the molecule and the substrate or product enantioselectivity of the process is difficult to determine due to the analytical problems discussed previously (Chapters 3 and 4). Thus, the stereoselectivity of the oxidative metabolism is difficult to discern without suitable analytical methods.

The first study on the pharmacokinetics of ibuprofen enantiomers in man (Van Giessen and Kaiser, 1975) reported a difference in the plasma elimination half-lives for the *R*- and *S*- enantiomers. A gas-liquid chromatographic method developed (Kaiser *et al.*, 1976) was utilised to study the stereoisomeric composition of the hydroxy and carboxy metabolites in urine after administration of the individual isomers, as well as the racemate. (*S*)-Hydroxyibuprofen was formed in enantiomeric excess, regardless of the stereochemical form of the administered drug i.e. racemate or either single enantiomer. However, it was not possible to determine whether this indicated stereoselectivity in oxidation or was a result of chiral inversion. The gas-liquid chromatographic method used here could not resolve the 2'*R*,2*S*- and 2'*S*,2*R*- isomers of carboxyibuprofen and this only permitted limited interpretation of the results obtained following administration of either the racemate or (*R*)-ibuprofen.

Lee *et al.*, (1985), demonstrated unequivocally the unidirectional nature of the chiral inversion process in man by studying the plasma kinetics of the enantiomers after separate administration of both the racemate and the individual enantiomers of ibuprofen. The extent of chiral inversion was estimated to be 63 % and there was no measurable extent of *S* to *R* inversion after administration of (*S*)-

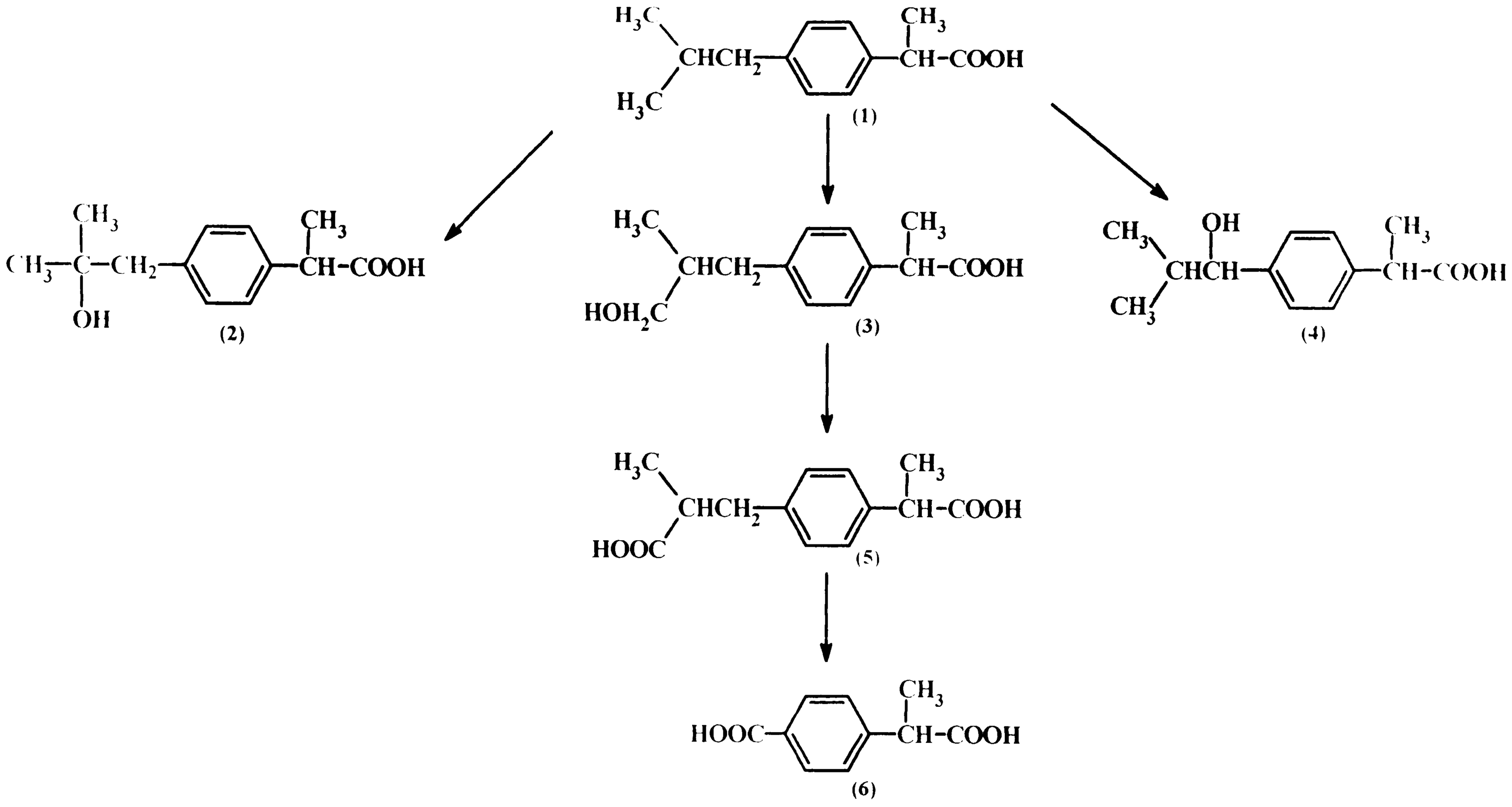


Figure 6.1: **Metabolic oxidation pathways of ibuprofen. (1 = ibuprofen; 2 = hydroxyibuprofen; 5 = carboxyibuprofen).**

ibuprofen. Comparison of the areas under the plasma-concentration versus time curves (AUC) following administration of the racemate showed lower AUCs than would be predicted by administration of the enantiomers separately. This was proposed to result from enantiomer-enantiomer mutual displacement in protein binding, although this was not examined in this study. Partial metabolic clearance by glucuronidation was also found to be greater for (*S*)- ibuprofen but whether this was due to inherent stereoselectivity in transformation or as a result of the enantioselective plasma protein binding could not be determined as unbound drug enantiomer concentrations were not measured (Evans, 1992), although *in-vitro* evidence seems to suggest stereoselective metabolism (El Mouelhi *et al.*, 1987).

Various other studies have similarly investigated the plasma pharmacokinetics of ibuprofen enantiomers either by administration of the racemate or the individual enantiomers (Jamali *et al.*, 1988; Cox *et al.*, 1988; Avgerinos and Hutt, 1990; Geisslinger *et al.*, 1990). In contrast with the findings of Lee *et al.*, (1985) and Van Giessen and Kaiser (1975), Jamali and co-workers (1988) found that elimination half-lives of both enantiomers to be similar and the enantiomeric plasma ratio (*S R*) increased only up to four hours post drug administration, thereafter decreasing to a constant value. A pre-systemic site of inversion, notably intestinal inversion was postulated to account for this observation. However, this argument was not supported by subsequent pharmacokinetic studies (Avgerinos and Hutt, 1990; Hall *et al.*, 1993) or *in-vitro* studies using animal tissues (Jeffrey *et al.*, 1991).

As a result of the difficulties involved in enantiospecific analysis, the majority of studies on ibuprofen disposition in the 1970s and 80s were carried out on a limited number of subjects. However the interindividual variability in the enantioselective pharmacokinetics of ibuprofen following the oral administration of 400 mg of the racemate to 24 healthy volunteers was reported by Avgerinos and Hutt (1990). Although the extent of inversion could not be calculated without the administration of individual enantiomers, examination of the percentage of the total AUC due to the *S*-enantiomer indicated that either the extent of chiral inversion showed little variation within the population group, or that the variability in

inversion was balanced by similar variability in alternative metabolic or excretory pathways. Similar lack of interindividual variability was also observed in the other determined pharmacokinetic parameters (Avgerinos and Hutt, 1990).

Being a drug of low hepatic extraction, the plasma protein binding of ibuprofen is a primary determinant of total drug clearance. This was clearly demonstrated in a human study involving 15 healthy volunteers (Lockwood *et al.*, 1983), where a non-linear relationship was found between AUC of total plasma concentration and dose, suggesting a change in plasma clearance. When unbound drug concentrations were measured, the AUC of the unbound drug was linearly related to dose, indicating dose-independent clearance of unbound drug and the cause of the non-linearity to be linked to changes in plasma protein binding. Subsequent studies have indicated an increase in the unbound fraction of both enantiomers was found to be the cause of the non-linearity between AUC and dose over the range of 200 - 1200 mg of the racemate, while intrinsic clearance was unchanged for both enantiomers (Evans *et al.*, 1990; Paliwal *et al.*, , 1993; Smith *et al.*, 1994) and emphasised the importance of determining unbound concentrations in pharmacokinetic studies of ibuprofen enantiomers.

As metabolic oxidation forms a major elimination pathway of ibuprofen, the stereoselectivity of the oxidative reactions need to be addressed. In one of the earliest studies by Kaiser *et al* (1976), (*S*)-hydroxyibuprofen was preferentially formed after administration of either racemic drug or (*R*)-ibuprofen. This could be a result of extensive chiral inversion, inherent stereoselectivity of the oxidation reaction (Baillie *et al.*, 1989) or as a result of stereoselective plasma protein binding (Evans, 1992). This question can be resolved if the partial metabolic clearance based on unbound concentrations, of each of the enantiomers of hydroxyibuprofen were determined. As a consequence of the methodological difficulties in resolving the diastereoisomeric forms of carboxyibuprofen, the stereoselectivity of this oxidation pathway remains largely answered. Studies based on the analytical method of Kaiser *et al.*, (1976) have suggested the possibility of stereoselective clearance of the (*S*)-ibuprofen via this route, although product stereoselectivity at

the newly formed chiral centre could not be ascertained (Baillie *et al.*, 1989; Smith *et al.*, 1994). However, there is some evidence that the *S*- configuration may predominate at this chiral centre, although the stereochemical configuration with respect to order of elution of the chromatographic peaks were not directly determined (Rudy *et al.*, 1990).

It is therefore apparent that any study of the enantioselective disposition of ibuprofen needs to address, the extent and variability of chiral inversion, stereoselectivity in plasma protein binding as well as the stereoselectivity of the major metabolic pathways. The majority of the studies discussed above have addressed some of these issues but a comprehensive study to obtain an overall picture of the relative importance of the various processes involved has not been reported in the literature.. With the use of the analytical methods developed in Chapters 2 , 4 and 5, the following section describes a single dose pharmacokinetic study in healthy young volunteers that examines the above issues.

6.2 Experimental

6.2.1 Clinical Study Protocol

(a) Inclusion criteria

Eight healthy young volunteers between the ages of 20 to 35 years (mean age of 28.1 $\pm$ 5.4, see Appendix 5 for volunteers' characteristics) were studied. The volunteers were required to give written consent before the study and underwent a thorough physical examination and routine biochemical tests before being recruited into the study. All aspects of the study were conducted according to the Helsinki declaration and ethical approval was obtained from the ethical committee of the School of Medicine and Dentistry, King's College London.

(b) Study Design

The volunteers were required to abstain from any medication, alcohol and beverages containing caffeine for 24 hours prior to the study. They were also required to fast from midnight of the study day until three hours post drug administration. Fluids were allowed up to 6 a.m. on the day of the study. Volunteers swallowed a single 400 mg tablet of racemic ibuprofen (Brufen^{R}) with a glass of 150 ml water. Blood samples (10 ml) were collected via an indwelling cannula at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0 3.5, 4, 6, 8, 10 and 24 hours post drug administration. The blood samples were collected in plain vacutainers and left to clot for two hours prior to centrifugation and separation of serum. Serum was stored frozen at -20°C until required for analysis. Volunteers were also required to empty their bladders prior to drug administration. Sequential urine samples were collected over the following time intervals; 0-2, 2-4, 4-6, 6-8, 8-10 and 10-24 hr, and the individual urine volumes were recorded. A pooled 24 hour sample was prepared from the individual samples,. and a 50 ml portion of each of the urine samples saved and frozen at -20°C until required for analysis. Serum albumin concentrations were determined as detailed in Chapter 5 and the individual data is presented in Appendix 5.

(c) Drug Analysis

The enantiomeric composition of ibuprofen in serum and urine was determined using the methods described in Chapter 2. Unbound ibuprofen enantiomer concentrations were determined using the methodology described in Chapter 5 for serum samples obtained at 0.5, 1.0, 2.0 , 3.0 and 4.0 hours. The urinary enantiomeric and diastereoisomeric composition of hydroxyibuprofen and carboxyibuprofen were determined using the methods described in Chapter 4.

6.2.2 Pharmacokinetic and data analysis

Pharmacokinetic analysis of the serum concentrations of the enantiomers were performed using the non-compartmental approach with the use of the TOPFIT curve fitting program (Instruction manual for TOPFIT pharmacokinetic curve fitting program). The terminal disposition constant (k_e) and elimination half life ($t_{1/2}$) for each serum concentration-time profile was calculated by linear regression of the log-linear portions of the serum concentration versus time profiles. The area under the curve (AUC) values from time 0 to ∞ for each serum profile were calculated from the time of administration to the last measurable serum concentration by the trapezoidal rule and were extrapolated to infinity using the last measurable serum concentration and k_e (Gibaldi and Perrier, 1982). Extrapolated areas were typically less than 2% of total AUC. The C_{max} and t_{max} values were determined by visual inspection of the data. Apparent total body clearance (CL) and volume of distribution (V_d) were calculated according to the following expressions:

$$CL/f = Dose/AUC_{0-\infty} \qquad \text{(eqn 6.2)}$$

$$V_d/f = Dose/k_e . AUC_{0-\infty} \qquad \text{(eqn 6.3)}$$

where f is the fraction of the dose reaching the systemic circulation. As the bioavailablity of orally administered ibuprofen is essentially unity (Hall *et al.*, 1993), f = 1 was assumed to be unity for all calculations.

The fraction of (*R*)-ibuprofen undergoing inversion (F_{inv}) was calculated from an examination of the stereochemical composition of the drug and both major metabolites, (free and conjugated) excreted in urine using the following equation:

$$F_{inv} = \frac{\%\ S \text{ metabolites} \ - \ \%\ S \text{ dose}}{\%\ R \text{ dose}} \qquad \text{eqn 6.4}$$

where % *S* dose and the % *R* dose are the percent of the administered dose in the *S* and *R* configuration respectively, i.e. 50%, and "% *S* metabolites" is the sum of the

molar amounts of metabolites excreted with the *S* configuration in the propionic acid moiety expressed as a percentage of that recovered.

Assuming all of a given metabolite formed appears in the urine, the formation clearances (CL_f) were calculated as:

$$CL_f = A_m/AUC_{parent} \qquad \text{(eqn 6.5)}$$

where A_m is the amount of metabolite recovered in urine and AUC_{parent} is the AUC of the enantiomer from which the metabolite was derived (Rudy *et al.*, 1991).

The unbound fraction (f_u) of each enantiomer was determined as follows:

$$\%\ f_u = \frac{\text{concentration in dialysate}}{\text{concentration in serum}} \times 100 \qquad \text{(eqn 6.6)}$$

(Knadler *et al.*, 1989). The mean of the five determinations for each individual serum profile were calculated and used in subsequent data analysis. The half life ($t_{1/2u}$) and elimination rate constant (k_{eu}) for each unbound concentration-time profile was calculated using the TOPFIT program based on linear regression of the log-linear portions of the unbound enantiomer concentration versus time curves profile. The AUC for unbound drug (AUC_u) was calculated as:

$$AUC_u = \%\ \text{unbound} \times AUC \qquad \text{(eqn 6.7)}$$

Total body clearance and volume of distribution based on unbound concentrations (CL_u and V_{du} respectively) were calculated as before:

$$CL_u = Dose/AUC_u \qquad \text{(eqn 6.8)}$$

$$V_{du} = CL_u/(k_{eu}) \qquad \text{(eqn 6.9)}$$

The mean pharmacokinetic parameters derived from the total and unbound serum data and urinary excretion data for the enantiomers of ibuprofen and

hydroxyibuprofen and the stereoisomers of carboxyibuprofen were tested for statistical significance using the Student's t-test for paired samples.

6.3 Results

6.3.1 Serum level data

The mean serum concentration versus time curves for both ibuprofen enantiomers following the administration of 400 mg of the racemate to eight healthy young volunteers are shown in Figure 6.2, and the individual serum level data is tabulated in Appendix 6. The pharmacokinetic parameters derived from the serum concentrations are presented in Table 6.1. Following administration of 400 mg of the racemate, the serum concentrations of the (*S*)-ibuprofen were greater than those of the *R* enantiomer after 2 hours post-dose. The (*S*)-ibuprofen serum levels also declined more slowly, and this is reflected by the significantly longer $t_{1/2}$ values ($p < 0.001$; Table 6.1). These findings are consistent with previously reported data (Van Giessen and Kaiser, 1975; Lee *et al.*, 1985; Cox *et al.*, 1988; Avgerinos and Hutt, 1990). The slower decline of the (*S*)-ibuprofen levels, results in a gradual enrichment of (*S*)-ibuprofen in serum (Figure 6.2).

The calculation of the clearance (CL) of (*S*)-ibuprofen requires a knowledge of the actual dose administered (eqn 6.2) but as a result of chiral inversion, the actual "dose" or systemic exposure of an individual to the *S* enantiomer is not known. However, an estimate of the "dose" can be made with the availability of F_{inv} values calculated from the urinary recovery data (see below) and the (*S*)-ibuprofen "dose" is calculated as:

$$\text{(S)-ibuprofen "dose"} = (1 + F_{inv})200 \text{ mg} \qquad \text{(eqn 6.10)}$$

The AUC, CL and other pharmacokinetic parameters also reflect differences between the individual enantiomers, although only CL, V_d and $t_{1/2}$ showed

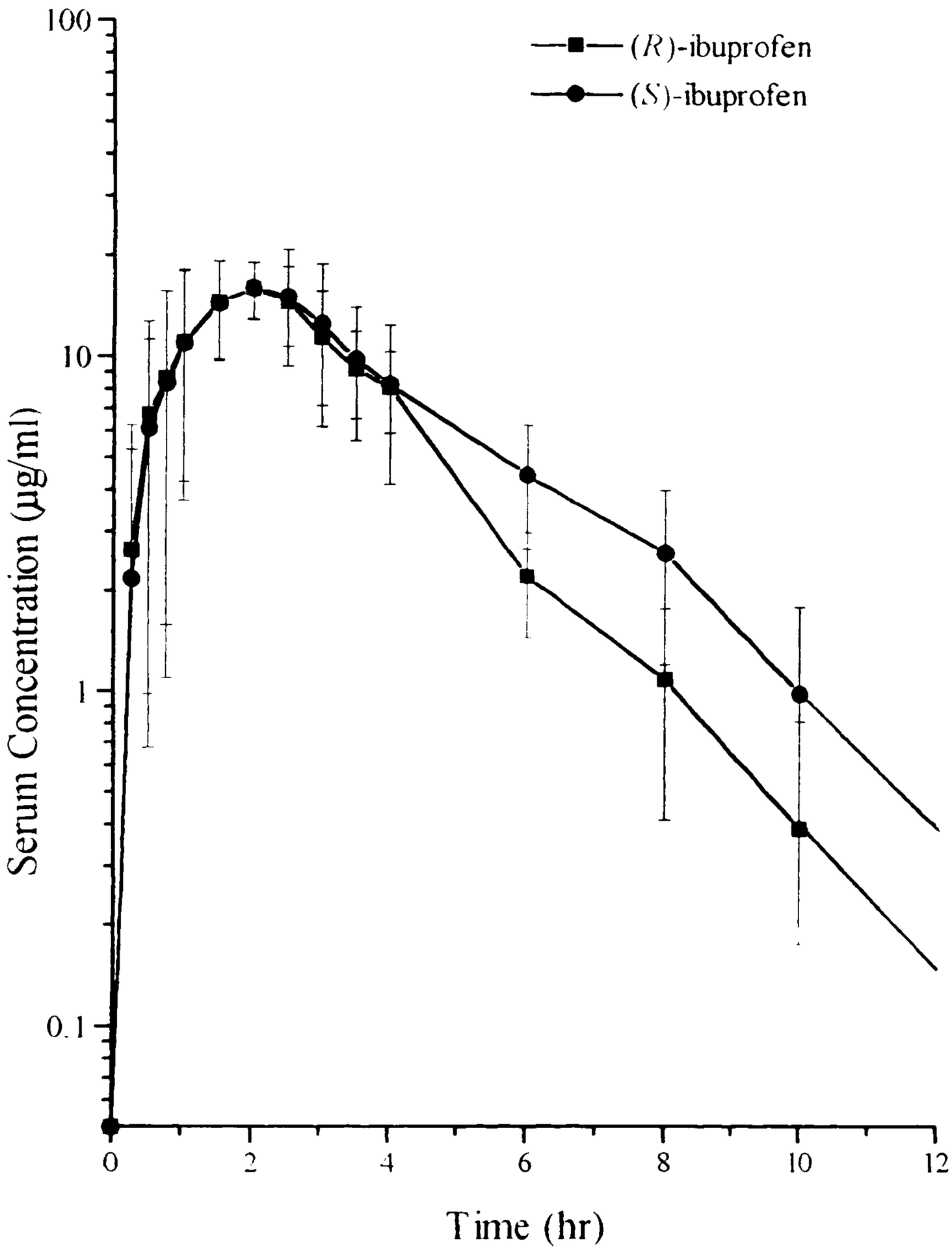


Figure 6.2: Mean ibuprofen enantiomer concentration-time curves following the oral administration of 400 mg of racemic drug to eight healthy young volunteers (mean ± sd).

Table 6.1 : Pharmacokinetic parameters of ibuprofen enantiomers following the oral administration of the racemic drug(400 mg) to eight healthy young volunteers.

Subject No:	Cmax (µg/ml)	Tmax (hr)	$t_{1/2}$ (hr)	AUC (µg/ml hr)	Cl/f (ml/min)	Vd/f (L)	Finv
				***R*)-ibuprofen**			
s1	17.7	2.0	1.2	53.0	62.9	6.4	0.65
s2	13.0	1.5	2.5	57.0	58.5	12.5	0.68
s3	19.5	2.0	1.2	66.9	49.8	5.1	0.68
s4	21.6	2.5	1.5	80.7	41.3	5.4	0.63
s5	13.6	2.5	1.1	49.0	68.0	6.2	0.6
s6	21.4	1.0	1.2	69.4	48.0	5.2	0.78
s7	12.2	2.0	1.6	53.7	62.1	8.8	0.78
s8	20.8	1.0	1.2	63.8	52.2	5.3	0.67
Mean	17.5	1.8	1.4	61.7	55.4	6.9	0.68
SD	4.0	0.6	0.5	10.5	9.0	2.6	0.07
CV%	22.7	32.8	32.1	17.1	16.2	37.4	9.5
				***S*)-ibuprofen**			
s1	16.7	2.0	1.7	52.3	105.2	15.2	
s2	15.2	1.5	3.0	75.3	74.4	19.6	
s3	14.8	2.0	1.9	58.7	95.4	15.5	
s4	26.2	2.5	2.1	120.0	45.3	8.2	
s5	21.6	2.5	2.2	94.2	56.6	10.7	
s6	23.2	1.0	2.2	91.0	65.2	12.2	
s7	12.6	2.0	1.9	54.9	108.1	17.7	
s8	20.0	1.0	2.5	53.6	103.9	22.5	
Mean	18.8	1.8	2.2	75.0	81.7	15.2	
SD	4.7	0.6	0.4	24.8	24.5	4.7	
CV%	25.1	32.8	19.7	33.0	30.0	31.1	
p value*	n.s.	n.s.	$p<0.001$	n.s.	$p<0.05$	$p<0.01$	

* Comparison between the means for the enantiomers of ibuprofen were carried out using Student's t-test for paired samples. (n.s. = $p > 0.05$).

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Table 6.2: Urinary excretion of ibuprofen and hydroxyibuprofen enantiomers and and carboxyibuprofen isomers following the administration of the racemic drug (400 mg) to eight healthy young volunteers (expressed as a percentage of dose).

		Mean	s.d.	Range
(*R*) - ibuprofen	free:	0.06	0.02	0.01 - 0.24
	conjugated:	1.09	0.31	0.66 - 1.59
	total	1.15	0.37	0.70 -1.82
(*S*) - ibuprofen	free:	0.38	0.11	0.31 - 0.56
	conjugated:	8.07	3.01	4.47 - 11.22
	total	8.45	3.19	4.70 - 13.86
(*R*) - hydroxyibuprofen	free:	1.96	0.53	1.22 - 2.81
	conjugated:	1.61	0.90	1.08 - 3.17
	total	3.57	1.24	2.64 - 5.46
(*S*) - hydroxyibuprofen	free:	5.40	1.86	3.41 - 7.85
	conjugated:	16.10	3.14	11.40 - 21.42
	total	21.50	3.70	16.99 - 27.05
carboxyibuprofen				
(2'*S*,2*R*)	free:	1.83	0.39	1.17 - 4.11
	conjugated:	0.95	0.80	0.55 - 1.49
	total	2.79	0.62	1.80 - 4.99
(2'*R*,2*R*)	free:	2.69	0.61	1.44 - 4.20
	conjugated:	2.27	0.78	2.35 - 2.80
	total	4.96	0.65	3.75 - 6.36
(2'*R*,2*S*)	free:	8.38	2.34	5.45 - 12.43
	conjugated:	8.07	2.16	5.21 -11.01
	total	16.45	4.17	12.68 -23.45
(2'*S*,2*S*)	free:	10.50	2.14	6.46 - 15.96
	conjugated:	9.35	2.53	7.14 - 11.88
	total	19.85	4.39	13.36 - 25.85
Total recovery		78.72	11.82	66.43 - 91.68

Note: Comparison between the means for the enantiomers of ibuprofen and hydroxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were carried out using Student's t test for paired samples. All means tested were found to be statistically significant($p < 0.01$).
Comparison between the means for the individual stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen respectively were done using a Student's t-test for paired samples.
The means tested were found to be statistically significant ($p < 0.05$).

Table 6.3: Formation clearances (ml/min) of ibuprofen glucuronides and total hydroxyibuprofen enantiomers and carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

Subject	Ibuprofen		Hydroxyibuprofen		Carboxyibuprofen		Carboxyibuprofen			
No:	*R*	*S*	*R*	*S*	2'*S*,2*R*+2'*R*,2*R*	2'*R*,2*S*+2'*S*,2*S*	2'*S*,2*R*	2'*R*,2*R*	2'*R*,2*S*	2'*S*,2*S*
s1	0.88	5.99	6.65	34.48	14.26	55.84	6.28	7.99	22.89	32.95
s2	0.87	5.50	3.49	11.33	6.47	21.96	2.13	4.34	9.17	12.79
s3	2.08	10.28	3.03	20.58	7.25	35.55	2.98	4.27	15.22	20.33
s4	0.89	6.18	3.01	17.99	8.37	23.74	2.91	5.46	11.46	12.28
s5	1.79	16.83	6.78	24.10	11.35	36.30	4.44	6.90	15.40	20.90
s6	1.49	14.26	2.82	31.32	6.18	55.51	1.88	4.29	26.80	28.71
s7	1.38	4.94	3.59	17.46	8.75	34.37	2.67	6.08	16.60	17.78
s8	0.82	3.64	2.47	12.45	6.26	19.69	1.78	4.49	9.90	9.79
mean	1.27	8.45	3.98	21.22	8.61	35.37	3.13	5.48	15.93	19.44
s.d.	0.48	4.82	1.72	8.33	2.86	14.08	1.53	1.41	6.21	8.13
CV %	37.95	57.04	43.31	39.28	33.26	39.81	48.72	25.65	38.98	41.83
p value*	p<0.005		p<0.0005		p< 0.001		p<0.00005		p< 0.05	

* Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were done using a Student's t-test for paired samples. (n.s.= p > 0.05).

Note: data for carboxyibuprofen are presented for both the individual stereoisomers and also for the sum of the stereoisomers produced from an individual isomer of ibuprofen, i.e. the two diasteroisomers 2'*S*,2*R* and 2'*R*,2*R* arise via oxidation of (*R*)-ibuprofen whereas the 2'*R*,2*S* and 2'*S*,2*R* arise from the oxidation of the *S*-enantiomer.

stereoselectivity with respect to formation of the alcohol and the metabolite appears to show substrate selectivity for conjugation. Examination of the formation clearances for the sum of the diastereoisomers of carboxyibuprofen arising from oxidation of either enantiomer of the drug indicates substrate stereoselectivity for (*S*)-ibuprofen (*S R* = 4.1). However, examination of the corresponding data for the formation of the individual stereoisomer indicates a reduced, but significant product stereoselectivity for the formation of a particular diastereoisomer. Thus, oxidation of ibuprofen to yield carboxyibuprofen shows substrate-product stereoselectivity with the predominantly formed diastereoisomer having the same configuration at the metabolically introduced centre as that in the substrate in both cases, i.e oxidation of (*R*)-ibuprofen preferentially yields (2'*R*,2*R*)-carboxyibuprofen, whereas (*S*)-ibuprofen preferentially yields (2'*S*, 2*S*)-carboxyibuprofen. It is also of interest to note that the product selectivity is greater in the case of the *R*-enantiomer than that for (*S*)-ibuprofen (2'*R*,2*R*/2'*S*,2*R* = 1.75; 2'*S*,2*S*/2'*R*,2*S* = 1.22; see Table 6.3). Such differences presumably reflect the orientation of the substrates to the active sites of the cytochrome P_{450} mediating the reaction.

The conjugation of carboxyibuprofen also appears to show some stereoselectivity, for example the mean ratios of free:conjugate for the four stereoisomers are: 1.9; 1.2; 1.0; and 1.1 for the 2'*S*,2*R*; 2'*R*,2*R*; 2'*R*,2*S*; 2'*S*,2*S* respectively (see Table 6.2) Thus, the 2'*R*,2*S*- stereoisomer appears to be a poorer substrate for conjugation than the other three carboxyibuprofen stereoisomers. The significance of this data is, however limited as at present the regioselectivity, i.e. the position of conjugation is unknown.

The extent of chiral inversion, F_{inv} is shown in Table 6.1. The mean value of 0.68 agrees well with previously reported values (Lee *et al.*, 1985; Rudy *et al.*, 1991; Smith *et al.*, 1994), calculated by either separate administration of the *R*-enantiomer, by the urinary excretion method, or by administration of a pseudoracemate. An interesting observation is the small interindividual variability of F_{inv}, consistent with the observations made previously based on indirect evidence (Avgerinos and Hutt, 1990).

6.3.3 Plasma protein binding and pharmacokinetic parameters based on unbound concentrations.

The plasma protein binding for each volunteer (expressed as % unbound) was determined by taking an average of the values obtained by analysing the equilibrium dialysate from five serum samples selected during the absorption and elimination phases of the serum concentration versus time curve (Table 6.4). Mean values of 0.25 and 0.51% were obtained for (*R*)- and (*S*)-ibuprofen respectively, and these compare favourably with values of 0.33 and 0.55 % obtained by Evans *et al.*, (1990) with a similar dose of racemate but analysed using a methodology based on the use the radiolabelled drug. The slight difference may be due to presence of radiolabelled impurities experienced when radiolabelled drugs are used in equilibrium dialysis (Evans *et al.*, 1990). The percentage unbound values between the enantiomers are statistically significant ($p < 0.0001$) using a Student's t-test for paired samples, indicating stereoselective binding in favour of the *R* enantiomer. The ratio of the unbound *S*/*R* concentration is 2.04, and is within the range of values of between 1.7 to 2.1 from previously reported in *in-vivo* studies (Evans *et al.*, 1990; Paliwal *et al.*, 1993; Smith *et al.*, 1994).

The pharmacokinetic parameters based on unbound concentrations (Table 6.5) showed enantioselective differences in $AUC_{u.}$, $t_{1/2u}$, and CL_u ($p < 0.001$), with the *S* enantiomer displaying a higher AUC_u, longer $t_{1/2u}$ and lower CL_u. The unbound metabolic clearance of (*R*)-ibuprofen is a result of the unbound clearance by inversion and non-inversion pathways, thus:

$$CL_U^R \quad CL_{other.u}^R + CL_{inv.u}^R \qquad \text{(eqn 6.11)}$$

The inversion clearance $CL_{inv.u}^S$ can be calculated as:

$$CL_{inv.u}^R = F_{inv}\, CL_U^R \qquad \text{(eqn 6.12)}$$

Table 6.4: Percentage unbound of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

				(*R*)-ibuprofen			
Subject	Sampling time (hr)						
No:	0.5	1.0	2	3	4	mean	s.d.
s1	0.15	0.13	0.27	0.23	0.32	0.22	0.08
s2	0.33	0.29	0.35	0.32	0.43	0.35	0.05
s3	0.31	0.26	0.33	0.24	0.34	0.29	0.05
s4	0.25	0.19	0.14	0.17	0.23	0.19	0.04
s5	0.30	0.20	0.27	0.15	0.22	0.23	0.06
s6	0.40	0.43	0.23	0.19	0.37	0.32	0.11
s7	0.17	0.21	0.26	0.17	0.16	0.20	0.04
s8	0.26	0.30	0.23	0.23	0.14	0.23	0.06
				(*S*)-ibuprofen			
s1	0.32	0.31	0.53	0.74	0.78	0.54	0.22
s2	0.52	0.57	0.62	0.61	0.30	0.52	0.13
s3	0.50	0.56	0.49	0.41	0.60	0.51	0.07
s4	0.41	0.43	0.28	0.33	0.21	0.33	0.09
s5	0.41	0.32	0.31	0.22	0.45	0.34	0.09
s6	0.51	0.60	0.51	0.40	0.40	0.49	0.08
s7	0.80	0.72	0.71	0.67	0.39	0.66	0.16
s8	0.60	1.20	0.49	0.74	0.41	0.69	0.31

Note: Average value for all determinations for (*R*)-ibuprofen is 0.25 (± 0.08) and that for (*S*)-ibuprofen is 0.51 (± 0.19). The means were tested for statistical significance using a Student's t-test for paired samples, $p < 0.0001$.

since a certain proportion of the unbound clearance of (*R*)-ibuprofen constitutes chiral inversion. Substituting equation 6.12 in 6.11 gives the following expression:

$$CL^{R}_{other,u} = (1 - F_{inv})CL^{R}_{u} \qquad \text{(eqn 6.13)}$$

The results of these clearance values are shown in Table 6.5. The inversion clearance for (*R*)-ibuprofen was predominant, accounting for 68% of the total unbound clearance for that enantiomer. A comparison of $CL_{other,u}$ of (*R*)-ibuprofen and CL_u for (*S*)-ibuprofen revealed that CL_u of (*S*)-ibuprofen was twice as large, indicating that clearance through the oxidative and glucuronidation pathways was stereoselective for the *S*- enantiomer.

The stereoselectivity in the unbound clearances of the (*S*)-ibuprofen is also reflected in the unbound formation clearances of the metabolites (Table 6.6). Comparison of the enantiomeric ratios (*S*:*R*) in the formation clearances based on total and unbound drug calculations results in reduced but still significant differences between the enantiomers for all metabolic pathways. Thus, the ratio of the formation clearances for ibuprofen glucuronides decreased from *S*:*R* = 6.7 to 3.1 when calculated from total and unbound enantiomer concentrations respectively. Similarly, the corresponding values for the hydroxy metabolite decreased from 5.3 to 2.4 and those for the sum of the carboxy diastereoisomers derived from a particular ibuprofen enantiomer decreased from 4.1 to 1.9. However, the values for the diastereoisomers derived from a single enantiomer of ibuprofen remained constant at 1.74 and 1.22 for the 2'*R*,2*R*/2'*S*,2*R* and 2'*S*,2*S*/2'*R*,2*S* respectively. This latter result is to be expected as the fraction unbound is taken into account in comparison of the ratios. Therefore, even though the relative stereoselectivity of the individual pathways is reduced when based on unbound clearance values, the data indicate inherent stereoselectivity of the metabolic pathways involved. Thus, the stereochemical composition of the metabolites found in urine arises as a result of stereoselectivity in both protein binding and metabolism. In addition, selectivity in

Table 6.5 : Pharmacokinetic parameters based on unbound concentrations of ibuprofen enantiomers in following oral administration of the racemic dose (400 mg) to eight healthy young volunteers.

Subject No:	% unbound	AUCu (mg/ml hr)	$t_{1/2\,u}$ (hr)	Vd_u (L)	CL_u (L/min)	$CL_{inv\,u}$ (L/min)	$CL_{other\,u}$ (L/min)
				(*R*)-ibuprofen			
s1	0.22	0.12	1.08	2673.1	28.59	18.58	10.01
s2	0.35	0.20	2.20	3182.6	16.71	11.36	5.35
s3	0.29	0.19	0.96	1429.5	17.18	11.68	5.50
s4	0.19	0.15	1.46	2748.0	21.74	13.70	8.04
s5	0.23	0.11	1.18	3021.7	29.58	17.75	11.83
s6	0.32	0.22	1.28	1663.4	15.01	11.71	3.30
s7	0.2	0.11	1.34	3600.8	31.04	24.21	6.83
s8	0.23	0.15	1.17	2301.1	22.72	15.22	7.50
mean	0.25	0.16	1.33	2577.5	22.82	15.53	7.29
sd	0.06	0.04	0.38	744.4	6.30	4.47	2.72
CV (%)	23.17	28.14	28.66	28.9	27.60	28.78	37.26
				(*S*)-ibuprofen			
s1	0.54	0.28	2.43	4097.2	19.47		
s2	0.52	0.39	2.73	3380.4	14.30		
s3	0.51	0.30	1.99	3222.9	18.71		
s4	0.33	0.40	1.57	1865.0	13.72		
s5	0.34	0.32	3.56	5132.6	16.65		
s6	0.49	0.45	1.65	1900.9	13.31		
s7	0.66	0.36	1.37	1942.3	16.38		
s8	0.69	0.37	1.43	1863.5	15.05		
mean	0.51	0.36	2.09	2925.6	15.95		
sd	0.13	0.05	0.77	1242.6	2.27		
CV (%)	25.41	15.29	36.66	42.5	14.25		
p value	$p < 0.001$	$p < 0.001$	$p < 0.05$	n.s.	$p<0.05$		

* Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R* and (*S*)-ibuprofen were carried out using Student's t-test for paired samples. n.s. = $p > 0.05$

Table 6.6: Formation clearances (L/min) based on unbound concentrations of ibuprofen glucuronides and hydroxyibuprofen enantiomers and the stereoisomers of carboxyibuprofen following oral administration of the racemic drug (400 mg) to eight healthy young volunteers.

Subject	Ibuprofen		Hydroxyibuprofen		Carboxyibuprofen		Carboxyibuprofen			
No:	*R*	*S*	*R*	*S*	2'*S*,2*R*+2'*R*,2*R*	2'*R*,2*S*+2'*S*,2*S*	2'*S*,2*R*	2'*R*,2*R*	2'*R*,2*S*	2'*S*,2*S*
s1	0.40	1.11	3.02	6.39	6.48	10.34	2.85	3.63	4.24	6.10
s2	0.35	1.68	1.41	3.47	2.62	6.73	0.86	1.75	2.81	3.92
s3	0.63	1.95	0.91	3.91	2.19	6.75	0.90	1.29	2.89	3.86
s4	0.39	1.17	1.31	3.42	3.65	4.51	1.27	2.38	2.18	2.34
s5	0.85	2.88	3.23	4.13	5.41	6.22	2.12	3.29	2.64	3.58
s6	0.43	1.70	0.81	3.77	1.77	6.61	0.54	1.23	3.22	3.45
s7	0.63	1.28	1.64	4.54	3.99	8.94	1.22	2.77	4.31	4.62
s8	0.39	0.90	1.17	3.06	2.96	4.85	0.84	2.12	2.44	2.41
mean	0.51	1.59	1.69	4.09	3.63	6.87	1.32	2.31	3.09	3.78
stdev	0.18	0.63	0.93	1.03	1.62	1.95	0.78	0.88	0.79	1.21
CV(%)	34.75	39.93	54.90	25.33	44.66	28.33	58.66	38.28	25.70	31.96
p value	p<0.0001		p<0.0001		p=0.005		p<0.0005		p=0.05	

* Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were done using a Student's t-test for paired samples (n.s = $p > 0.05$).

See note in Table 6.3.

renal excretion may also contribute to the observed differences, however at present, this cannot be determined.

6.4 Discussion

As can be seen from the results, the pharmacokinetics of ibuprofen enantiomers are complicated by the fact that enantioselectivity occurs in plasma protein binding, metabolic oxidation and glucuronidation, and that chiral inversion forms a major clearance pathway of the *R* enantiomer. As a result of the reported enantiomer-enantiomer displacement and non-linearity in protein binding (Paliwal *et al.*, 1993), a comparison of the results obtained above with previously published data is difficult. Also a wide range of study designs, based on administration of either the racemate or individual enantiomers, and different dosages further complicates comparisons.

Estimation of the extent of chiral inversion can be carried out in a number of ways, depending on whether the individual enantiomers, racemate or a pseudoracemate are administered. When (*R*)-ibuprofen is administered, F_{inv} can be estimated by direct comparison of the AUCs obtained for the *R* and *S* enantiomers (Baillie *et al.*, 1989). Alternatively, the *R* and *S* enantiomers can be given to the same individual on separate occasions and F_{inv} calculated by comparing the AUCs as follows:

$$F_{inv} \quad \frac{AUC_{R,S}}{AUC_{S,S}} \qquad \text{(eqn 6.14)}$$

where $AUC_{R,S}$ and $AUC_{S,S}$ are the AUCs of the *S*- enantiomer after administration of the *R*- and *S*- enantiomers respectively (Lee *et al.*, 1985). An underlying assumption of this method is that the kinetics of (*S*) ibuprofen are time independent between subsequent study periods, an assumption which needs to be addressed (Rudy *et al.*,

1991; Smith *et al.*, 1994). As a result of this, the administration of a pseudoracemate involving $(S)_{D4}$-ibuprofen was employed (Rudy *et al.*, 1991). Based on serum levels of $(S)_{D4}$-ibuprofen, CL and AUC of (*S*)- ibuprofen without the contribution from chiral inversion can be calculated, and hence F_{inv} can be estimated. This approach is not possible using an unlabelled racemic dose. When racemic doses are administered F_{inv} can be conveniently calculated by determining the stereochemical content of the drug and its metabolites in urine. The results obtained in the present study using this method, fall within the range of 0.52 to 0.74 reported in the literature (Geisslinger *et al.*, 1990 ;Lee *et al.*, 1985; Hall *et al.*, 1993; Smith *et al.*, 1994).

Because of the limits of sensitivity imposed by the non-radiolabelled method for the determination of ibuprofen enantiomers in equilibrium dialysis, the unbound concentrations could not be determined for all serum samples. As such five samples were chosen along the absorption, at the peak and during the elimination phases of the serum concentration versus time curves. The AUC_u was then calculated as mean percent unbound x AUC, as shown in equation 6.7 . This method of calculation is valid as the serum concentrations were within the limits where plasma protein binding was constant, i.e. unbound concentrations were linearly related to total serum concentrations (Figure 6.3). This approach was adopted in preference over that of estimating AUC_u directly based on only five unbound levels as had been done previously (Evans *et al.*, 1990) as more errors will be introduced in the calculation of unbound AUC based on only five unbound concentrations.

Although the CL, V_d and formation clearances of the metabolites show stereoselective differences between the individual enantiomers, whether these changes are a result of the stereoselectivity in protein binding can only be ascertained by evaluating the corresponding parameters based on unbound concentrations. While the total CL of (*S*)-ibuprofen was significantly greater than that of the *R*-enantiomer, the reverse situation was observed when unbound clearance values were examined, i.e. CL_u, $R > S$, indicating that the higher total CL

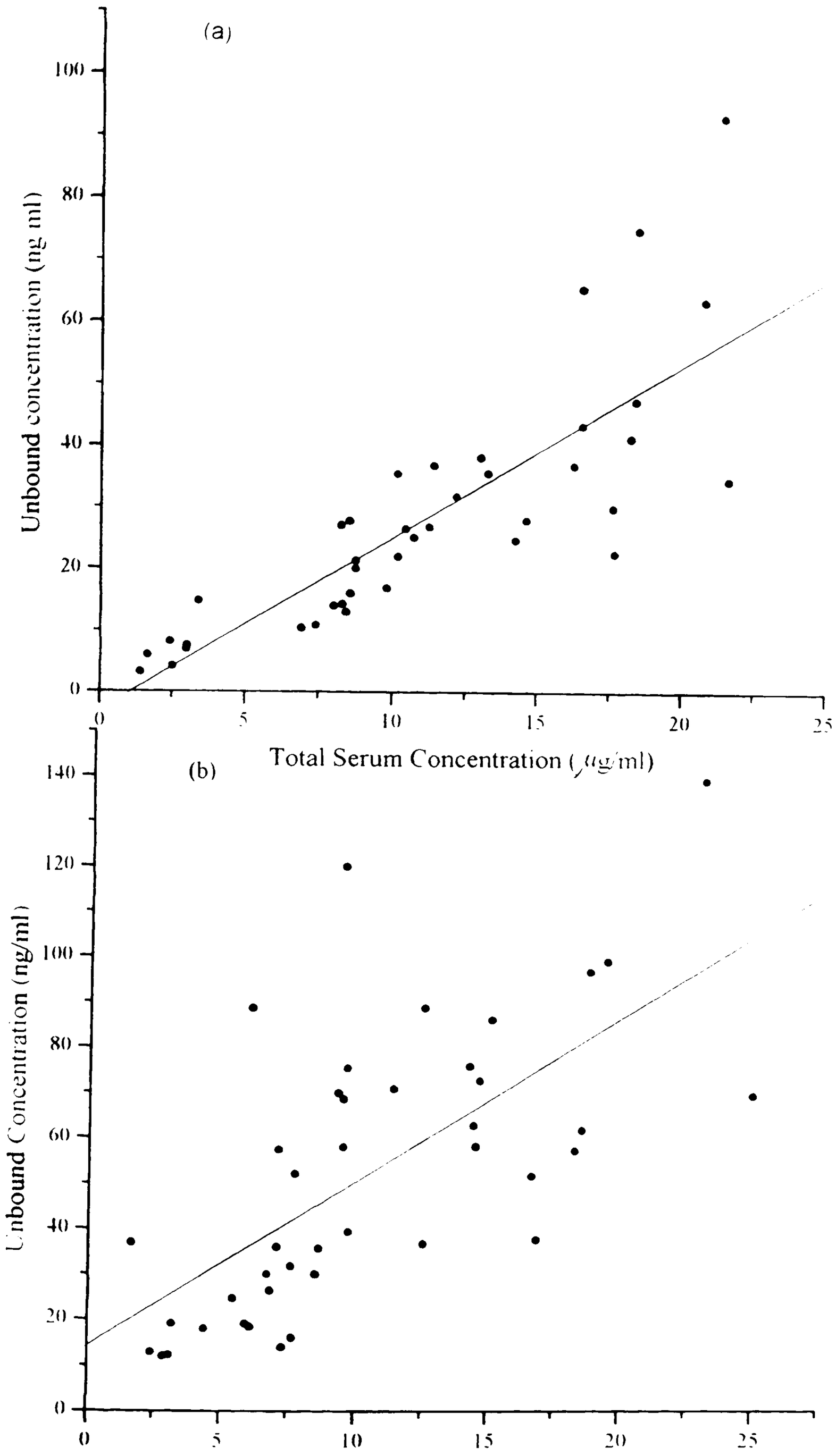

Figure 6.3: Plot of unbound concentrations versus total (bound and unbound) serum concentrations of (a) (*R*)-ibuprofen and (b) (*S*)-ibuprofen in serum samples following the oral administration of racemic drug (400 mg) to eight healthy young volunteers.

for the *S* enantiomer was due to higher unbound concentrations. The Cl_u of (*R*)-ibuprofen is mainly due to clearance by inversion ($CL_{inv.u}$) which is twice as large as clearance by alternative pathways ($CL_{other.u}$). The overall picture can be represented in Figure 6.4.

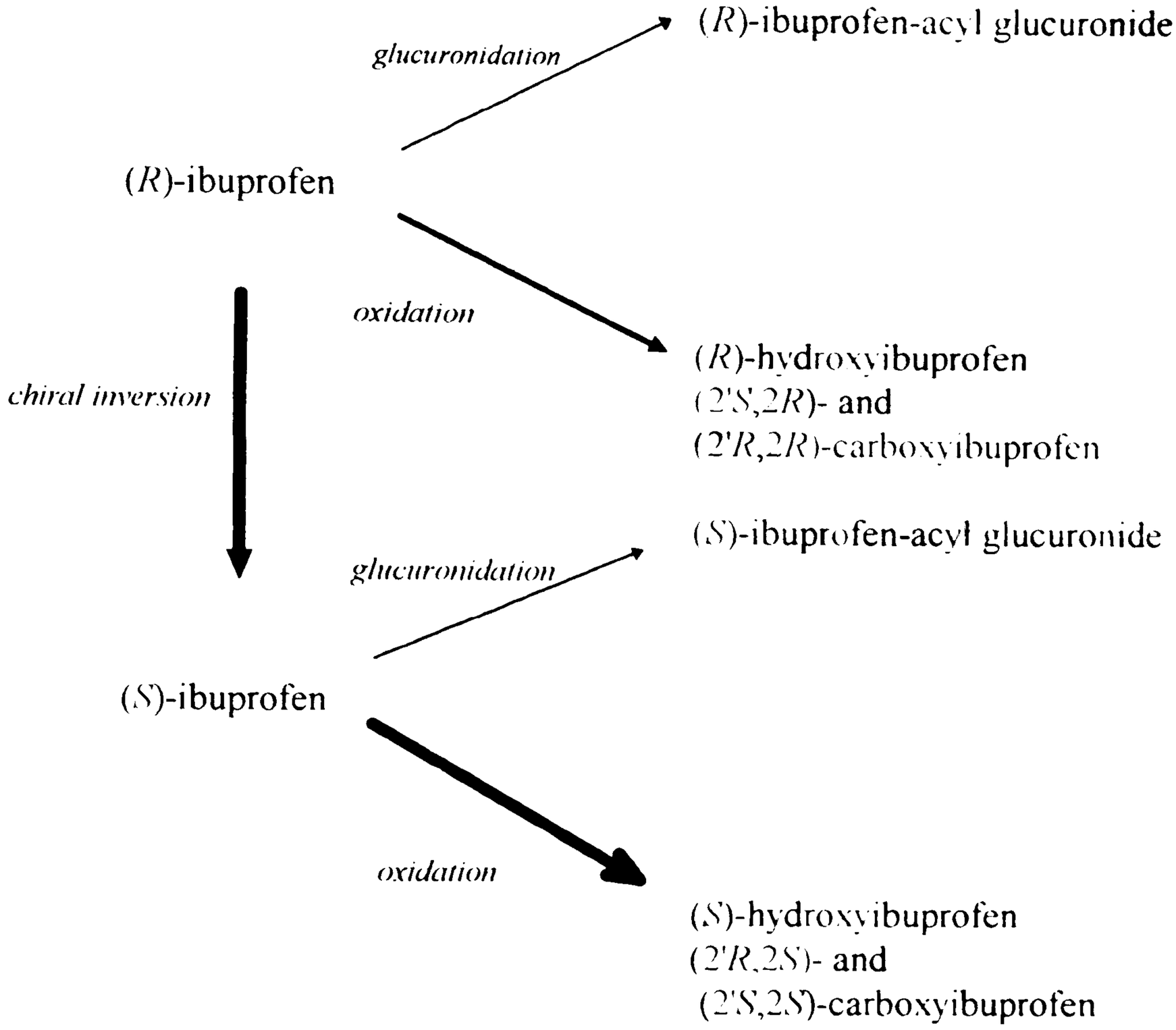

Figure 6. 4 : Schematic representation of the enantioselective clearance of ibuprofen. The line thickness of the arrows indicate relative importance of the pathway.

Based on the above model, one would expect that the metabolites with the *S*-configuration in the propionic acid moiety to predominate in urine. This is reflected in the amounts of "*S* metabolites" excreted in urine as well in the higher formation clearances of all the "*S* metabolites". These findings contradict those of Rudy *et al*

(1991), who reported that the magnitude of CL_{inv} and CL_{other} of (*R*)-ibuprofen to be of the same magnitude. However *in-vitro* data using rat hepatocytes (Xiaotao and Hall, 1993) and subsequent reports by the same authors and others (Rudy *et al.*, 1995; Smith *et al.*, 1994) support the above finding that CL_{inv} is the main route of clearance for (*R*)-ibuprofen. The central importance of plasma protein binding is again illustrated in the case of the AUC and AUC_u values. While there was no difference in the AUC for both enantiomers, the AUC_u was significantly higher for the *S*-enantiomer (Table 6.5). The mean V_d for the *S*- enantiomer was also significantly higher than that of the *R* antipode. That this is again a consequence of stereoselective protein binding is apparent from the following expression:

$$V_d = V_p (1 + R_{E/I}) + V_T (f_u / f_T) \qquad \text{(eqn 6.15)}$$

where V_p is the plasma volume, $R_{E/I}$ is the ratio of plasma binding protein in the extracellular fluid to plasma, V_T is the volume of extravascular tissue and f_u and f_T are the fractions of unbound drug in plasma and tissue respectively. Assigning normal physiological values 1.4, 3.0L and 30L to $R_{E/I}$, V_p and V_T respectively to equation 6.15, then,

$$V_d = 7.2 + 30 (f_u / f_T) \qquad \text{(eqn 6.16)}$$

(Oie and Tozer, 1979; Lin *et al.*, 1987; Evans, 1992). As tissue binding is of minor importance with ibuprofen, it is therefore clear that the larger V_d of the *S*- enantiomer is most probably a result of the higher unbound fraction. This is corroborated by the fact that this enantiomeric difference disappears when f_u is not taken into consideration as with the case of V_{du} i.e. the unbound V_d are not significantly different between the two enantiomers (Table 6.5).

The formation clearances of the metabolites of ibuprofen also show stereoselectivity for the *S*-enantiomer. Although this is clearly consistent with higher clearances for (*S*)-ibuprofen, these differences could again be caused by stereoselectivity in protein binding. However, this was not the case as the unbound

formation clearances were also significantly different, indicating inherent enantioselectivity in functional oxidation and glucuronidation. A comparison between the total and unbound formation clearances for the metabolites also reveal that the magnitude of the stereoselective differences were smaller with unbound formation clearances, again revealing the influence of plasma protein binding on disposition, which would be expected for a low hepatic extraction drug (Rowland and Tozer, 1989).

6.5 Conclusion

Stereoselective differences were observed in the pharmacokinetics of the enantiomers of ibuprofen following the administration of the racemic drug to healthy young volunteers. Stereoselective protein binding was also evident, with higher unbound *S*- enantiomer concentrations and this stereoselectivity explains the differences observed in V_d and AUC_u between the enantiomers. Clearance by inversion was the major pathway for (*R*)-ibuprofen and the clearance by metabolic processes (oxidation and glucuronidation) was stereoselective for the *S*-enantiomer, being twice as large as the corresponding value for the *R*-enantiomer. The formation clearances of the metabolites were also stereoselective for (*S*)-ibuprofen, although these processes were inherently stereoselective and did not arise as a result of higher unbound *S* enantiomer concentrations. As regards the clearances of the individual carboxyibuprofen isomers, it is clear that the stereochemistry of the substrate influences the stereochemistry of the product at the newly formed chiral centre. Thus, for (*S*)-ibuprofen, the preferred product was (2'*S*,2*S*)-carboxyibuprofen while for (*R*)-ibuprofen, the preferred product was (2'*R*,2*R*)-carboxyibuprofen. The results of this single dose study emphasises the important role played by plasma protein binding on the disposition of a highly protein bound drug with a low hepatic extraction ratio.

CHAPTER 7

Enantioselective Pharmacokinetics of Ibuprofen in the Elderly

7.1 Introduction

As a class of compounds, the NSAIDs are extensively bound to plasma proteins and have low hepatic extraction, i.e. the intrinsic clearance is low compared to hepatic blood flow. As only the unbound fraction is available for clearance, conditions which affect the plasma protein binding of these drugs will affect of their pharmacokinetics and disposition (Lin *et al.*, 1987). As is apparent from the results presented in the previous chapter, the unbound fraction of (*R*)- and (*S*)-ibuprofen are 0.25 and 0.51 % respectively. With such a high extent of binding small changes in binding affinity may lead to substantial changes in the unbound fraction, and thus significantly affect the pharmacokinetics, disposition and pharmacological activity.

Many physiological and pathophysiological conditions affect plasma protein binding. These include ageing, loss of renal function or disease, hepatic disorders, stress, surgery, pregnancy, burns and drug interactions (Wallace and Verbeeck, 1987). Of particular interest here is the effect of ageing, as the NSAIDs are widely used in the elderly population (Walt *et al.*, 1986; Johnson *et al.*, 1993). Ageing affects plasma protein binding in two ways: firstly, the serum concentrations of albumin, the major protein to which acidic drugs bind, decreases with age. Secondly, differences in binding may also be a result of changes in the binding characteristics of the albumin (Dawling and Crome, 1989). There is also evidence that neonatal albumin differs in biochemical properties from that of adults (Wallace *et al.*, 1977) and changes in plasma protein binding occur.

Apart from affecting plasma protein binding, ageing also affects drug disposition through other mechanisms. With increasing age, body fat increases with a simultaneous decrease in body water (Woodhouse and Wynne, 1987). Thus highly lipid soluble drugs like lignocaine and diazepam tend to have an increased V_d in the elderly. Conversely, water soluble drugs like antipyrine would have a reduced V_d giving rise to higher plasma concentrations (Vestal *et al.*, 1975). Of greater

importance is the drug metabolising capacity of the liver. The hepatic clearance of a drug, CL_h, can be represented as:

$$CL_h = Q \times E \qquad \text{(eqn 7.1)}$$

where Q is the liver blood flow and E is the extraction ratio of the drug. Clearance of drugs which have a high hepatic extraction ratio is dependent on hepatic blood flow and thus, the decrease in blood flow in the aged affects the clearance of drugs like propranolol and morphine (Woodhouse and James, 1990). The intrinsic metabolic capacity of Phase I oxidation is also reduced with age. Thus, the systemic clearance of antipyrine is reduced in elderly subjects, accompanied with a 45 % decrease in $t_{1/2}$ (O'Malley *et al.*, 1971; Posner *et al.*, 1987). Compared to other physiological functions, renal function undergoes the most significant decrease with age, which is , estimated to be about 0.62% a year (Ritschel *et al.*, 1993). Drugs which are principally cleared by renal mechanisms are therefore most affected e.g. digoxin and gentamicin (Tregaskis and Stevenson, 1990).

Studies of the influence of age on the pharmacokinetics and disposition of the 2-APAs are scarce, and do not give a consistent picture. Albert *et al.*, (1984) reported the lack of effect of ageing on the pharmacokinetics on ibuprofen, but the clearance of ketoprofen and ibuprofen were reported to be decreased (Greenblatt *et al.*, 1984; Advenier *et al.*, 1983). That these discrepancies in the findings may be as a result of measuring only total drug concentrations, rather than both free and bound concentrations was highlighted by Upton *et al* (1984), who reported that measuring only total concentrations masks an increase in the unbound fraction together with a concomitant decrease in unbound clearance of naproxen. Clearly, studies in which only total drug concentration are measured offer limited information in these situations (Rudy *et al.*, 1995). An additional problem with the above studies on ibuprofen and ketoprofen is that the enantiomeric composition of the material in plasma was not taken into account.

The effect of ageing on the stereoselective pharmacokinetics of chiral drugs administered as racemates has also been poorly studied. As ageing affects various pharmacokinetic parameters which exhibit stereoselectivity e.g. protein binding, hepatic metabolism and renal elimination, it is conceivable that the disposition of chiral substances will exhibit differences in their stereoselective mechanisms in the elderly. In a study of the oral clearance of propranolol enantiomers in young and elderly volunteers, the elderly had a lower oral clearance for both enantiomers but no stereoselective effects were observed (Zhou *et al.*, 1992). Therefore, the decreased β-adrenergic blocking effect of the drug in the elderly is not due to changes in the stereoselective metabolism of the more active (-)-*S*-enantiomer. To date, differences in stereoselective drug disposition in the aged population have has only been reported for hexobarbital and mephobarbital. The clearance of *d*-hexobarbital was not age-related whereas that of *l*-hexobarbital was decreased two-fold in the elderly. However, as pharmacological activity resides only in the *d*-enantiomer, the pharmacological response was not affected (Chandler *et al.*, 1988). With mephobarbital, an age and gender-related effect was observed. The (*R*)-enantiomer was cleared more rapidly than the (*S*)- antipode in all cases. The oral clearance of the *R*- enantiomer in young men was higher than that observed inboth young females and elderly men. However , there were no significant differences between young and elderly women, or between elderly men and women in clearance. Thus the clearance of (*R*)-mephobarbitone is both age and gender related (Hooper and Qing, 1990). The object of the present investigation is to examine the pharmacokinetics of ibuprofen enantiomers following the oral administration of the racemic drug to healthy elderly volunteers and compare the data obtained to that presented in Chapter 6 in a group of young volunteers. During the course of these investigations, a report concerning the effect of ageing on the enantioselectivity of ibuprofen disposition has been published (Rudy *et al.*, 1995). The results of the present investigation will be compared with those of the study by Rudy *et al.*, (1995).

7.2 Experimental

7.2.1 Clinical Study Protocol

(a) Inclusion criteria

Eight healthy elderly volunteers between the ages of 65 to 80 years (mean age of 69.5 $\pm$ 4.4, see Appendix 5 for volunteer characteristics) were studied. The volunteers were required to give written informed consent prior to the study and underwent a thorough physical examination and routine biochemical tests before being recruited into the study. All aspects of the study were conducted according to the Helsinki declaration and ethical approval was obtained from the ethical committee of School of Medicine and Dentistry, King's College London.

(b) Study Design

The volunteers were required to abstain from any medication, alcohol and beverages containing caffeine 24 hours prior to the study. They were also required to starve from midnight prior to the study day until three hours post drug administration. Fluids were allowed up to 6 a.m. on the study day. Volunteers swallowed a single 400 mg tablet of racemic ibuprofen (Brufen^{R}) with a glass of 150 ml water. Blood samples of 10 ml were collected via an indwelling cannula at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0 3.5, 4, 6, 8, 10 and 24 hours post drug administration. The blood samples were collected in plain vacutainers and left to clot for two hours prior to centrifugation and separation of serum. Serum was stored frozen at -20°C until required for analysis. Volunteers were also required to empty their bladders prior to drug administration. Sequential urine samples were collected over the following time intervals; 0-2, 2-4, 4-6, 6-8, 8-10 and 10-24 hr, and the individual urine volumes were recorded. A pooled 24 hour sample was prepared from the individual samples and a 50 ml portion of each of the urine samples saved

and frozen at -20°C until required for analysis. Serum albumin concentrations were determined as detailed in Chapter 5 and the individual data is presented in Appendix 5.

7.2.2 Drug Analysis

The enantiomeric composition of ibuprofen in serum and urine was determined using the methods described in Chapter 2. Unbound concentrations of ibuprofen enantiomers were determined using the methods described in Chapter 5. The urinary enantiomeric and diastereoisomeric composition of hydroxyibuprofen and carboxyibuprofen were determined using the methods described in Chapter 4.

7.2.3 Pharmacokinetic and data analysis

Pharmacokinetic analysis of the serum and urinary concentrations were carried out as described for the young volunteers in section 6.2.2. The pharmacokinetic parameters derived from the total and unbound serum data and urinary excretion data for ibuprofen, carboxyibuprofen and hydroxyibuprofen were compared with those obtained for the young volunteers and tested for statistical significance using the Student's t-test for independent samples.

7.3 Results

7.3.1 Serum level data

The mean (*R*)- and (*S*)-ibuprofen serum concentration versus time plots are shown in Figure 7.1 and the individual serum level data are tabulated in Appendix 16. The pharmacokinetic parameters calculated from these data are presented in Table 7.1.

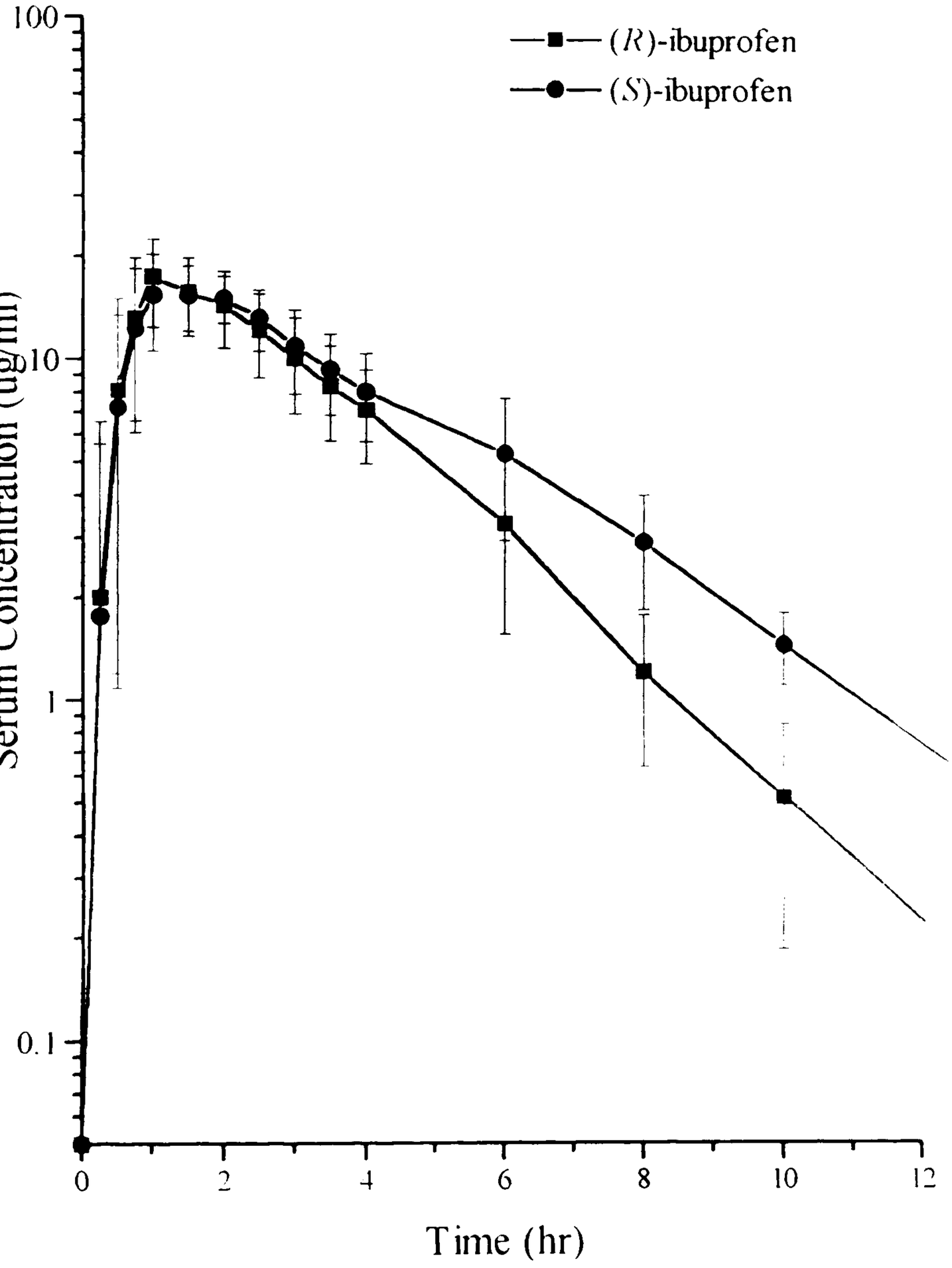

Figure 7.1 Mean ibuprofen enantiomer serum concentration-time curves following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers (mean ± sd).

Table 7.1: Pharmacokinetic parameters of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

Subject No:	Cmax (μg/ml)	Tmax (hr)	$t_{1/2}$ (hr)	AUC (μg/ml hr)	CL (ml/min)	Vd (L)	Finv
			(*R*)-ibuprofen				
es1	17.7	2.0	1.2	53.0	62.9	6.4	0.65
es2	20.8	1.5	1.7	80.0	41.7	6.1	0.66
es3	23.1	1.0	1.6	71.9	46.3	6.6	0.77
es4	17.8	1.0	2.2	68.6	48.6	9.1	0.60
es5	15.8	2.0	2.0	68.1	49.0	8.3	0.71
es6	10.6	0.8	1.5	45.9	72.7	9.5	0.64
es7	16.8	1.5	1.5	53.4	62.4	8.1	0.57
es8	20.2	1.0	2.0	73.3	45.5	8.0	0.63
Mean	18.4	1.2	1.7	64.5	53.4	7.8	0.64
SD	4.0	0.4	0.3	11.8	10.7	1.2	0.1
CV%	22.0	33.7	16.8	18.3	20.1	15.1	11.0
Y vs E p value**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
			(S)-ibuprofen				
es1	20.2	1.0	2.2	81.5	63.8	12.3	
es2	18.2	1.5	2.6	77.8	71.2	16.0	
es3	21.4	1.0	2.2	83.4	70.8	13.7	
es4	17.1	2.5	4.1	104.0	51.4	18.0	
es5	13.6	2.0	2.3	61.1	93.4	18.9	
es6	15.4	2.0	2.8	93.5	58.4	14.3	
es7	18.0	1.5	2.4	97.4	53.7	11.1	
es8	16.4	1.0	2.4	62.3	87.2	17.9	
Mean	17.5	1.6	2.6	82.6	68.7	15.3	
SD	2.5	0.6	0.6	15.6	15.2	2.9	
CV%	14.3	36.0	23.1	18.8	22.1	18.8	
p value*	n.s	n.s.	p<0.005	n.s.	n.s	p<0.0001	
Y vs E** *p* value	n.s	n.s	n.s	n.s	n.s	n.s	

* Comparison between the means of the enantiomers of ibuprofen were carried out using a Student's t-test for paired samples. (n.s.= $p > 0.05$).

** Comparison between respective means for young and elderly volunteers were carried out using a Student's t-test for independent samples (n.s. = $p > 0.05$).

Following the oral administration of 400 mg racemic ibuprofen serum concentrations of the *R*- and *S*-enantiomers were similar to those obtained with the young volunteers. The difference in $t_{1/2}$ values between the enantiomers were also statistically different in the elderly ($p < 0.005$), with the *S*-enantiomer having a significantly longer $t_{1/2}$. Similar differences in CL and V_d were also observed between the individual enantiomers. However, the difference in CL did not achieve statistical significance in the case of the elderly volunteers. Comparison of the values of the various pharmacokinetic parameters between the young and elderly group did not reveal any age related differences (Table 7.1).

7.3.2 Urinary excretion data

The urinary excretion data for ibuprofen and its metabolites for the elderly group are summarised in Table 7.2 and the individual data tabulated in Appendices 17 to 25. The calculated fractional inversion and formation clearances are tabulated in Tables 7.1 and 7.3. The total amount of drug related material recovered in the 0 - 24 hour urine is 84.1 (± 7.9) %. The hydroxy and carboxy metabolites formed 27.7 and 46.0 % of the administered dose excreted in urine. Unchanged ibuprofen, accounting for 9.7% of the administered dose was excreted mainly as the acyl glucuronide (9.2%; Table 7.2). As with the young group, ibuprofen was excreted mainly as the *S*-enantiomer (8.8%) with an *S*/*R* recovery ratio = 9.4. Stereoselective clearance was also observed with the hydroxyibuprofen and the carboxyibuprofen metabolites as reflected by the respective formation clearances, which were significantly greater for the metabolites derived from (*S*)-ibuprofen compared to those of the *R*- enantiomer (Table 7.3). The urinary recovery of the *S*-hydroxy metabolite showed marked stereoselectivity compared with that of the *R*-antipode (*S*/*R* = 5.1) and preferential conjugation of the *S*-enantiomer was also evident (ratio conjugated/free: *S* = 2.9; *R* = 1.0). Thus, the substrate stereoselectivity for the formation of hydroxyibuprofen and preferential stereoselectivity for conjugation observed in the young volunteers were also observed in the elderly group. The ratio (*S*/*R*) of the formation clearances of hydroxyibuprofen was found to be 4.2, which

Table 7.2: Urinary excretion of ibuprofen and hydroxyibuprofen enantiomers and carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers (data expressed as a percentage of dose).

		Mean	s.d.	Range
(*R*) - ibuprofen	free:	0.04	0.02	0.02 - 0.07
	conjugated:	0.89	0.33	0.68 - 1.44
	total:	0.93	0.34	0.43 -1.48
(*S*) - ibuprofen	free:	0.48	0.09	0.31 - 0.56
	conjugated:	8.32	2.12	5.27 - 12.06
	total:	8.79	2.17	5.66 - 12.58
(*R*) - hydroxyibuprofen	free:	2.31	1.04	0.99 - 3.49
	conjugated:	2.24	0.96	1.17 - 3.86
	total:	4.56	1.73	2.17 - 7.35
(S) - hydroxyibuprofen	free:	5.86	2.21	3.71 - 9.26
	conjugated:	17.28	5.00	9.53 - 24.03
	total:	23.14	5.54	15.03 - 32.53
carboxyibuprofen				
(2'*S*,2*R*)	free:	2.30	3.74	0.79 - 3.49
	conjugated:	1.07	0.88	0.25 - 2.57
	total:	3.37	0.96	1.53 - 4.49
(2'*R*,2*R*)	free:	3.07	4.51	1.44 - 4.20
	conjugated:	2.55	0.87	0.78 - 3.89
	total:	5.62	1.08	3.59 - 7.25
(2'*R*,2*S*)	free:	10.41	4.30	6.27 - 15.78
	conjugated:	6.74	3.80	2.51 - 11.83
	total:	17.15	5.58	14.70 - 20.20
(2'*S*,2*S*)	free:	10.45	4.39	5.85 - 15.86
	conjugated:	9.38	5.13	2.27 - 21.49
	total:	19.83	5.65	16.36 - 16.86
Total recovery		84.14	7.92	69.28 - 96.11

* Comparison between the means for the enantiomers of ibuprofen and hydroxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were carried out using Student's t-test for paired samples and were found to be statistically significant for all species ($p > 0.005$)
Comparison between the means for the individual stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen respectively were done using a Student's t-test for paired samples
The means tested were found to be statistically significant ($p < 0.005$) for the stereoisomers derived from (*R*)-ibuprofen but were not significant for those derived from (*S*)-ibuprofen ($p > 0.05$)
Note: Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples and were found to be not statistically significant ($p > 0.05$) for all species.

Table 7.3 : Formation clearances of (ml/min) of ibuprofen glucuronides and total hydroxyibuprofen enantiomers and carboxyibuprofen stereoisomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

Subject	Ibuprofen		Hydroxyibuprofen		Carboxyibuprofen		Carboxyibuprofen			
No:	*R*	*S*	*R*	*S*	2'*S*, 2*R*+2'*R*, 2*R*	2'*R*, 2*S*+2'*S*, 2*S*	2'*S*, 2*R*	2'*R*, 2*R*	2'*R*, 2*S*	2'*S*, 2*S*
se1	0.68	8.87	5.58	26.00	7.07	26.28	3.10	3.98	12.08	14.20
se2	0.99	13.46	4.32	30.44	9.41	35.12	2.82	6.59	16.34	18.78
se3	0.63	5.08	3.14	12.70	7.44	23.76	2.22	5.21	12.09	11.66
se4	0.78	6.18	4.45	24.75	10.47	40.02	4.40	6.07	18.63	21.40
se5	0.74	5.45	2.37	13.69	8.53	26.11	2.52	6.02	13.78	12.33
se6	1.06	5.57	5.02	10.28	11.34	28.45	4.89	6.44	10.06	18.38
se7	1.08	7.25	6.13	20.69	8.30	29.67	3.39	4.91	14.04	15.63
se8	1.81	7.18	6.32	18.92	12.66	35.32	4.81	7.85	16.52	18.80
mean	0.97	7.38	4.67	19.68	9.40	30.59	3.52	5.88	14.19	16.40
s.d.	0.38	2.75	1.39	7.13	1.95	5.64	1.05	1.18	2.82	3.48
CV %	39.2	37.3	29.8	36.2	20.8	18.4	29.8	20.1	19.9	21.2
p value*	p<0.0005		p<0.0005		p<0.00001		p<0.0005		n.s.	
Y vs E** p value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were carried out using Student's t-test for paired samples (n.s. = p > 0.05).

** Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples (n.s. = p > 0.05)

was not significantly different from that found in the young volunteers (*S/R* 5.3; Tables 6.3 and 7.3).

As with the young, the formation clearances for the carboxyibuprofen diastereoisomers arising from the oxidation of the respective *R*- and *S*- enantiomers of ibuprofen indicate substrate stereoselectivity for the latter (*S/R* = 3.3). For the formation of the individual stereoisomers, substrate-product stereoselectivity with the predominantly formed diastereomer having the same absolute configuration at the newly formed chiral centre, i.e. oxidation of (*R*)-ibuprofen preferentially yields (2'*R*,2*R*)-carboxyibuprofen while (*S*)-ibuprofen preferentially forms (2'*S*,2*S*)-carboxyibuprofen. However, comparison of the product stereoselectivity between the young and elderly for the *R*- enantiomer (2'*R*,2*R*/2'*S*,2*R* = 1.75 for the young, 1.67 for the elderly), and for the (*S*)-antipode (2'*S*,2*S*/2'*R*,2*S* = 1.22 for the young, 1.15 for the elderly) showed reduced stereoselectivity, such that the difference for the latter did not achieve statistical significance.

The extent of chiral inversion, F_{inv} is tabulated in Table 7.1. The mean value of 0.64 is similar to that of 0.68 obtained with the young group. The small interindividual variability of F_{inv} observed in the young volunteers is also evident here.

7.3.3 Plasma protein binding and pharmacokinetic parameters based on unbound concentrations.

The main differences between the young and the elderly groups are with the plasma protein binding and pharmacokinetic parameters based on unbound levels, the values of which are presented in Tables 7.4, 7.5 and 7.6. The mean percentage unbound values for the (*R*)- and (*S*)-ibuprofen are 0.30 and 0.76 % respectively, the difference being statistically different ($p < 0.001$), an observation also found with the young volunteers (Chapter 6). However, a direct comparison of the protein binding between the young and elderly group revealed an age-related effect. Whilst

Table 7.4: Unbound fraction (expressed as a percentage) of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

				(*R*)-ibuprofen				
Subject	Sampling time (hr)							
No:	0.5	1.0	2	3	4	mean	s.d.	CV(%)
es1	0.54	0.24	0.25	0.19	0.21	0.29	0.14	50.73
es2	0.35	0.31	0.19	0.17	0.14	0.23	0.09	39.77
es3	0.35	0.40	0.48	0.41	0.40	0.41	0.05	11.38
es4	0.13	0.15	0.27	0.60	0.29	0.29	0.19	64.73
es5	0.16	0.19	0.20	0.24	0.17	0.19	0.03	16.65
es6	0.28	0.49	0.51	0.30	0.45	0.41	0.11	26.89
es7	0.24	0.26	0.21	0.37	0.20	0.25	0.07	26.62
es8	0.16	0.47	0.31	0.48	0.37	0.36	0.13	36.95
				(*S*)-ibuprofen				
es1	0.76	1.11	1.03	0.63	0.28	0.76	0.33	43.60
es2	0.73	0.93	0.94	1.19	0.65	0.89	0.21	23.66
es3	0.86	1.05	0.96	0.86	0.60	0.87	0.17	19.42
es4	0.47	0.54	0.40	0.58	0.51	0.50	0.07	13.76
es5	0.67	0.58	0.40	0.56	0.64	0.57	0.10	18.27
es6	0.77	1.13	1.82	1.24	0.65	1.12	0.46	41.06
es7	0.48	0.46	0.49	0.55	0.35	0.47	0.07	15.34
es8	0.38	1.34	0.90	0.91	0.91	0.89	0.34	38.27

Note: Average value for all determinations for (*R*)-ibuprofen is 0.30 (± 0.12) and that for (*S*)-ibuprofen is 0.76 (± 0.30). The means were tested for statistical significance using Student's t-test for paired samples, $p < 0.0001$).
The average values for the unbound fractions of (*R*) and (*S*)-ibuprofen were compared between the young and elderly groups using Student's t-test for independent samples: $p > 0.05$ for (*R*)-ibuprofen and $p \leq 0.0001$ for (*S*)-ibuprofen.

the unbound fraction of the *R*-enantiomer was similar for both the young (0.25 ± 0.08) and elderly (0.30 ± 0.12), the elderly showed a significantly higher fraction for (*S*)-ibuprofen (young, 0.51 ± 0.19; elderly 0.76 ± 0.30; $p < 0.0001$).

The pharmacokinetic parameters based on unbound concentrations (Table 7.5) showed the same enantioselectivity between (*R*)- and (*S*)- ibuprofen as found with the young volunteer group, with differences in AUC_u, $t_{1/2u}$, and CL_u (see Table 7.5 for p values) between the *R*- and *S*- enantiomers. A statistical comparison between the young and elderly again reveal age-related differences in stereoselectivity. Although there were no differences in the unbound pharmacokinetic parameters of (*R*)-ibuprofen between the two groups, major differences were observed in the case of the *S*-enantiomer for AUC_u and CL_u, indicating that the elderly have a poorer clearance of the active (*S*)-ibuprofen (CL_u, young 15.9 L/min; elderly 9.7 L/min).

The age-related differences in the unbound clearance of (*S*)-ibuprofen are also reflected in the unbound formation clearances of the oxidative metabolites derived from the *S*-enantiomer (Table 7.6). While there were no age-related differences in the unbound formation clearances of the oxidative metabolites derived from (*R*)-ibuprofen, the unbound formation clearances for (*S*)-hydroxyibuprofen, (2′*R*,2*S*)- and (2′*S*,2*S*)-carboxyibuprofen for the elderly were significantly lower ($p < 0.05$) which indicates that clearance through the major metabolic pathways are reduced in the elderly. Clearance through direct glucuronidation of ibuprofen, a relatively minor pathway, tends towards lower unbound clearances for both enantiomers in the elderly but these differences did not achieve statistical significance.

A close examination of the unbound formation clearances of the individual carboxyibuprofen diastereoisomers also reveal some age related differences. In the young group, substrate related product selectivity was observed,. but the difference was markedly reduced in the elderly. The differences in the unbound formation

Table 7.5: Pharmacokinetic parameters based on unbound concentrations of ibuprofen enantiomers following the oral administration of the racemic drug (400 mg) to eight healthy elderly volunteers.

Subject No:	% unbound	AUCu (mg/ml hr)	$t_{1/2\,u}$ (hr)	V_{du} (L)	CL_u (L/min)	$CL_{inv,u}$ (L/min)	$CL_{other,u}$ (L/min)
(*R*)-ibuprofen							
es1	0.29	0.16	0.98	1793.5	21.05	11.79	9.26
es2	0.23	0.18	1.56	2446.8	18.12	11.96	6.16
es3	0.41	0.29	1.70	1664.3	11.31	8.71	2.60
es4	0.29	0.20	1.84	2669.3	16.76	10.05	6.70
es5	0.19	0.13	2.02	4505.5	25.76	18.29	7.47
es6	0.41	0.19	1.43	2193.0	17.71	11.34	6.38
es7	0.25	0.13	2.60	5620.7	24.97	14.23	10.74
es8	0.36	0.26	1.97	2154.5	12.63	7.96	4.67
mean	0.30	0.19	1.76	2881.0	18.54	11.79	6.75
s.d.	0.08	0.06	0.47	1416.4	5.22	3.29	2.53
cv (%)	27.09	30.39	26.93	49.2	28.18	27.89	37.47
Y vs E** *p* value	n.s	n.s	n.s	n.s	n.s	n.s	n.s
(*S*)-ibuprofen							
es1	0.76	0.62	1.14	828.6	8.40		
es2	0.89	0.69	2.15	1487.6	7.99		
es3	0.87	0.73	1.62	1140.5	8.13		
es4	0.50	0.52	3.80	3374.4	10.26		
es5	0.57	0.35	3.79	5370.5	16.37		
es6	1.12	1.05	2.49	1125.4	5.22		
es7	0.47	0.46	2.67	2642.7	11.43		
es8	0.89	0.55	2.12	1798.6	9.80		
mean	0.76	0.62	2.47	2221.0	9.70		
s.d.	0.23	0.21	0.95	1533.0	3.27		
cv (%)	30.02	34.09	38.27	69.0	33.73		
p value*	$p < 0.0005$	$p < 0.0005$	$p < 0.05$	n.s.	$p < 0.001$		
Y vs E** p value	$p < 0.0001$	$p < 0.01$	n.s.	n.s.	$p < 0.001$		

* Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were carried out using Student's t-test for paired samples. n.s = $p > 0.05$.

** Comparison between respective means for young and elderly volunteers were carried out using Student's t-test for independent samples. n.s. = $p > 0.05$.

Table 7.6. : Formation clearances of (L/min) based on unbound concentrations of ibuprofen glucuronide and total hydroxyibuprofen enantiomers and carboxyibuprofen stereoisomers following the oral administration of racemic drug (400 mg) to eight healthy elderly volunteers

Subject	Ibuprofen		Hydroxyibuprofen		Carboxyibuprofen		Carboxyibuprofen			
No:	*R*	*S*	*R*	*S*	2'*S*, 2*R*+2'*R*, 2*R*	2'*R*, 2*S*+2'*S*, 2*S*	2'*S*, 2*R*	2'*R*, 2*R*	2'*R*, 2*S*	2'*S*, 2*S*
se1	0.31	1.19	2.53	3.50	3.21	3.54	1.41	1.81	1.63	1.91
se2	0.40	1.21	1.72	2.74	3.75	3.16	1.12	2.63	1.47	1.69
se3	0.10	0.65	0.49	1.64	1.16	3.06	0.35	0.81	1.56	1.50
se4	0.27	0.73	1.52	2.91	3.58	4.70	1.50	2.08	2.19	2.51
se5	0.39	1.63	1.26	4.09	4.52	7.80	1.33	3.19	4.11	3.68
se6	0.30	0.52	1.42	0.95	3.22	2.64	1.39	1.83	0.93	1.71
se7	0.65	1.23	3.67	3.52	4.97	5.04	2.03	2.94	2.39	2.66
se8	0.37	1.05	1.31	2.78	2.62	5.19	1.00	1.62	2.43	2.76
mean	0.35	1.03	1.74	2.77	3.38	4.39	1.27	2.11	2.09	2.30
s.d.	0.15	0.37	0.96	1.03	1.17	1.68	0.48	0.78	0.97	0.74
CV %	44.6	36.0	55.3	37.3	34.6	38.3	38.0	36.7	46.2	32.0
p value*	$p<0.0005$		$p<0.05$		n.s.		$p<0.005$		n.s.	
Y vs E** p value	n.s.	n.s.	n.s.	$p<0.05$	n.s.	$p<0.05$	n.s.	n.s.	$p<0.05$	$p<0.05$

* Comparison between the means of the enantiomers of ibuprofen and hydroxyibuprofen and that of the individual stereoisomers of carboxyibuprofen and the stereoisomers of carboxyibuprofen derived from (*R*)- and (*S*)-ibuprofen were carried out using Student's t-test for paired samples (n.s. = $p>0.05$)

** Comparison between respective means for young and elderly volunteers carried out using Student's t-test for independent samples (n.s. = $p>0.05$).

clearances between the 2'*S*,2*R*- and 2'*R*,2*R*- diastereoisomers in the young and that in elderly are similar (Table 7.6). However, the difference in unbound formation clearances between the 2'*R*,2*S*- and 2'*S*,2*S*- diastereoisomers which was significant in the young ($p < 0.005$) with the 2'*S*2*S*- diastereoisomer being the preferred product, disappeared in the elderly. The formation of both the 2'*R*,2*S*- and (2'*S*,2*S*)-diastereoisomers were markedly lower in the elderly, but a greater decrease was recorded for the 2'*S*2*S*- isomer, ageing resulting in reduced formation of the diastereoisomers arising from (*S*)-ibuprofen and also a reduction in product stereoselectivity.

7.4 Discussion

Previous studies comparing the pharmacokinetics of ibuprofen in the young and elderly were mainly based on non-stereoselective drug determinations, and have reported conflicting results. Ibuprofen clearance was found to be slightly but significantly lower in elderly men than in young male subjects (Greenblatt *et al.*, 1984). In another study, however trends towards a higher total clearance, but lower clearance and volume of distribution based on unbound levels, were found in the elderly, although these did not reach statistical significance (Albert *et al.*, 1984).

Two major factors appear to influence the total clearance of ibuprofen, protein binding and unbound or intrinsic clearance. A consideration of the influence of these two processes are further complicated when the stereochemistry of the drug is taken into account, with the additional problem of chiral inversion of (*R*)- ibuprofen In the case of the *R*-enantiomer, there appears to be no significant age associated effects in protein binding, total unbound clearance and inversion clearance. However, in the case of (*S*)-ibuprofen, protein binding and unbound clearance appear to operate in opposite directions which results in no apparent differences with age when total drug enantiomer concentrations are determined. Thus, the increase in fraction unbound with age (young, 0.51; elderly 0.76) negates the observed decrease in unbound clearance (young, 15.9 L/min; elderly, 9.7 L/min).

A similar observation has also been reported for the related NSAID naproxen (Upton *et al.*, 1984).

The decrease in the unbound clearance of (*S*)- ibuprofen with age can either be due to reduced metabolic function and/or reduced renal elimination of the readily hydrolysable glucuronide conjugates (Upton *et al.*, 1984). However, the parallel reduction in the respective unbound formation clearances of the oxidative metabolites derived from (*S*)-ibuprofen, but not the extent of excretion of the metabolites over 24 hours strongly suggests that the former is the most probable cause. As the clearance of the *S*-enantiomer by oxidation represents the main metabolic pathways, this stereoselective reduction in clearance of the (*S*)-ibuprofen but not that of the *R*-antipode suggests that the more active metabolising enzyme(s) or sites of the enzyme(s) are principally affected with ageing. This age-related effect also influenced product stereoselectivity in the case of (2′*R*,2*S*)- and (2′*S*,2*S*)- carboxyibuprofen, where the product stereoselectivity for the formation of (2′*S*,2*S*)- isomer observed in the young was lost in the elderly. In the study of ageing on the stereoselctive disposition of hexobarbital, it was postulated that separate isoenzymes may be involved in the metabolism of the (+)- and (-)- enantiomers and that ageing alters the activity of the isoenzymes responsible for (-)- hexobarbital metabolism (Chandler *et al.*, 1988).

In contrast to the age-related differences in clearance of the major oxidative metabolites, clearance via the minor glucuronidation pathway did not exhibit any significant differences, although there was a trend towards a lower clearance for both enantiomers in the elderly. It has been suggested that Phase II glucuronidation is not generally affected by ageing (Mooney *et al.*, 1985) and if this assumption holds true in the case of ibuprofen, then the small reduction may be attributed to reduction in renal clearance, which is generally observed in the elderly. As glucuronidation represents only a minor elimination pathway, any slight decrease in renal clearance will not manifest itself clearly, thus explaining the slightly lower but not significantly different clearance in the elderly. With ketoprofen, a 2-APA principally cleared by glucuronidation, clearance was reduced in elderly subjects

(Advenier *et al.*, 1985; Dennis *et al.*, 1985). Whether this decrease was caused by a decrease in glucuronidation or reduced renal elimination could not be ascertained but the weight of evidence in these studies seems to suggest renal causes.

In the elderly, increases in the unbound fraction of drugs such as diazepam, phenytoin and warfarin have been well documented (Tregaskis and Stevenson, 1990). With the 2-APAs, a significant increase in the unbound fraction of naproxen has been reported (Upton *et al.*, 1984). The results presented here indicate that ageing stereoselectively increased the unbound fraction of (*S*)-ibuprofen, but not that of (*R*)-ibuprofen. Whilst it is generally accepted that the increase in fraction unbound may be due to lower serum albumin concentrations in the elderly (Wallace and Verbeeck, 1987), this does not seem to be the case here as the serum albumin concentrations (see Appendix 8) measured did not show appreciable differences between the two groups, an observation also reported for naproxen where the small difference in serum albumin could not account for the large difference in protein binding (Upton *et al.*, 1984). Also the unbound fraction of (*R*)-ibuprofen was not significantly affected, which would be expected if lower serum albumin had been the cause. It has also been suggested that the increase in unbound (*S*)-ibuprofen in the elderly may be due to the displacement of protein binding by the oxidative metabolites (Rudy *et al.*, 1995). If this is the case then the difference with the elderly would be because of higher amounts of oxidative metabolites generated compared to the young group, leading to increased displacement. However as the formation clearances of the metabolites are actually decreased in the elderly, as reflected in the lower unbound formation clearances, this explanation seems unlikely.

In contrast to the observed differences in plasma protein binding, there was no significant change in the V_{du}. Generally one would expect changes in plasma protein binding to be accompanied by parallel changes in V_{du} provided that the fraction unbound in tissue does not alter. The quantitative relationship between V_d and protein binding is given by (see Chapter 6, section 6.4) :

$$V_d = 7.2 + 30\ (f_u / f_T) \qquad \text{(eqn 7.2)}$$

Thus from equation 7.2, it is apparent that V_d depends on f_u as well as f_T. For a highly protein bound drug like ibuprofen, f_u approaches zero and V_d is approximately 7.2L, which is essentially the volume of distribution of albumin, and changes in f_u will not have a significant effect on the value of V_d (Lin *et al.*, 1987), as illustrated in the results of this study.

The fact that plasma protein binding and unbound clearance vary in opposite directions with age tend to present a false picture of the underlying pharmacokinetic changes that occur in the elderly. This illustrates the limited utility of performing pharmacokinetic studies based solely on total drug concentrations. Since it is the unbound concentrations that elicit pharmacological effects, the increase in unbound (*S*)-ibuprofen levels suggest that the elderly may experience an enhanced response and side-effects to the drug as compared to the young. The implications of this together with the effects of other pathophysiological conditions commonly associated with the elderly are discussed in Chapter 8.

While this study was in progress, a report of the enantioselective disposition of ibuprofen in the elderly with and without renal impairment appeared in the literature (Rudy *et al.*, 1995). Some of the reported findings support the observations of the present study. A stereoselective increase in the unbound fraction of (*S*)-ibuprofen was reported in both groups of elderly subjects. However, the decrease in the unbound clearance of (*S*)-ibuprofen was only significant in the renally impaired group, but not in the healthy elderly subjects, unlike the findings of this study and that of Upton *et al* (1984), for naproxen. Strangely, the reduction in unbound drug enantiomer clearances was not accompanied by the expected decrease in the formation clearances of the major oxidative metabolites as demonstrated in the present study. This anomaly was, however not addressed (Rudy *et al.*, 1995).

7.5 Conclusions

The differences between the pharmacokinetics (*R*)- and (*S*)-ibuprofen observed in the young were also observed in the elderly. A comparison between the young and elderly showed that ageing appeared to have a minimal effect on the pharmacokinetics based on total drug concentrations. However, when unbound concentrations were used, various age-related changes in the stereoselective disposition were observed. The fraction unbound was higher for the (*S*)-, but not (*R*)-ibuprofen in the elderly. The unbound clearance of the *S*-enantiomer was reduced in the elderly and its effect on plasma clearance was masked by the increase in unbound fraction. This decrease in unbound clearance was reflected in the decrease in unbound formation clearances of the major oxidative metabolites, while the minor glucuronidation pathway was not significantly affected.

Thus the data presented above indicate that age associated differences in the pharmacokinetic properties of ibuprofen do occur and indicate the significance of determining both free and total drug levels together with their enantiomeric composition. The significance of these observations will be examined in Chapter 8.

CHAPTER 8

General Discussion and Conclusions

8.1 Introduction

The studies described in this thesis were initiated to investigate the enantioselective disposition of ibuprofen in the young and elderly. There are many reports of the enantioselective disposition of ibuprofen in healthy young subjects using various experimental designs, but very few of them have actually attempted to obtain a comprehensive picture of the relative magnitudes of the various dispositional processes. This is especially true in the case of the major metabolite, carboxyibuprofen, where analytical difficulties have prevented the elucidation of the stereoselectivity in the formation of the four stereoisomers. These issues have been addressed in this study.

Despite the fact that ibuprofen and related NSAIDs are frequently administered to elderly patients for the relief of inflammation and pain associated with rheumatoid arthritis (Walt *et al.*, 1986; Johnson *et al.*, 1993), there is a curious lack of attention of the influence of ageing on drug disposition. Studies based on non-stereospecific assays on ibuprofen (Greenblatt *et al.*, 1984; Albert *et al.*, 1984), ketoprofen (Advenier *et al.*, 1983) and naproxen (Upton *et al.*, 1984) have reported disparate findings of the effects of ageing and it was hoped that a study such as this would present a clearer picture.

8.2 Enantioselective analysis of ibuprofen and its metabolites

The indirect approach to enantioselective analysis offers an economical alternative towards an otherwise expensive procedure. The method used in this study, being based on a reversed phase C_{18} column is relatively cheap to operate and is very robust, the same column being used for the urine and serum samples in both the young and elderly groups. Although not generally favoured because of long reaction times, derivatization with CDI and (*R*)-NEA in the presence of HOBT is essentially quantitative and has not posed problems like racemization, which has been known to occur with the mixed anhydride method (Hutt *et al.*, 1994). This

reaction approaches that of a "universal" method applicable for the indirect analysis of the profens, an idea put forward by Spahn (1987). Using the same column and with minor modifications to the acetonitrile content in the mobile phase, other profens e.g. ketoprofen, carprofen, tiaprofenic acid were successfully resolved (unpublished observations). (*R*)-1-(Naphthen-1-yl)ethylamine being a strong fluorophore affords an extra level of sensitivity when used with a fluorescence detector. Thus with the better mass detectability afforded by a semi-microbore column, the methodology was adapted for the determination of unbound enantiomer concentrations. Although unbound concentrations of less than 5 ng/ml could not be determined, the use of chiral amines with a stronger fluorescence properties will help to improve detectability. Reagents based on anthracene or naphthylamines, with strong electron donating substituents, are ideal for this purpose. With increased sensitivity, future studies on plasma protein binding of chiral acids can be carried out without resorting to the use of radiolabelled compounds. The advantages of this approach are obvious, as unbound enantiomer concentrations can be determined directly without the hazards associated with the handling of radioactive substances.

The main drawback of the indirect method, especially if strong fluorophores or chromophores are used, is that excess chiral amine, when injected into the HPLC system will yield a large reagent peak that inevitably causes interference problems of co-elution. However, a silica solid-phase extraction cartridge serves as a good scavenger as the excess amine will bind to the acidic silanol groups, while allowing the neutral diastereomeric amides to flow through.

In addition to being commercially available in high purity, both the *R*- and *S*-enantiomers of NEA are available. This adds flexibility to the approach as the order of elution of the enantiomers can be controlled by judicious choice of the chiral amine. In a reversed phase system, using (*R*)-NEA as derivatizing reagent, the order of elution of the diastereomeric amides is (*R*)-before (*S*)-ibuprofen derivative. When (*S*)-NEA is used, the order of elution is reversed. In the method developed, (*R*)-NEA was chosen as derivatizing reagent in order to elute the (*R*)-ibuprofen

derivative first as (*R*)-ibuprofen concentrations were expected to be lower than those of the *S*-enantiomer in serum, and earlier elution would reduce the effects of band broadening on a smaller peak. Under normal phase conditions, the order of elution is again the opposite of that for reversed phase systems (Hutt *et al.*, 1994). This flexibility is a useful aid in the assignment of stereochemical configuration to chromatographic peaks in a group of closely related compounds.

Sensitivity of analytical procedures are often reported as minimum detection limits, which is not a true reflection of the ability of the method to quantify in a reproducible manner at those concentrations reported. A more useful method is to report the minimal quantifiable limit, which is the minimum concentration at which the procedure is able to quantify an analyte in a reproducible manner, as had been done with the method developed. In spite of the difficulties in comparing analytical sensitivity with those previously reported, the analytical method presented is more sensitive than those bioanalytical HPLC procedures based on CSPs (Nicoll-Griffith *et al.*, 1988; Geisslinger *et al.*, 1989; Li *et al.*, 1993; Naidong and Lee, 1994; de Vries *et al.*, 1994), equally if not more sensitive than those based on indirect approaches with UV detection (Lee *et al.*, 1984; Avgerinos and Hutt, 1987; Mehvar *et al.*, 1988), and equally sensitive with the method based on fluorescent detection (Lemko *et al.*, 1993). Examination of sample chromatograms presented in the literature indicates that the resolution between the diastereomer derivatives, and from co-extracted endogenous substances, obtained using the method presented in Chapter 2 were superior to those methods cited above. In addition peak shape was more symmetrical than those obtained from use of CSPs.

Separation of the stereoisomers of carboxyibuprofen by the indirect approach did not give good resolution and separation of the four stereoisomers was not possible within a reasonable run time due to co-elution of two of the stereoisomer derivatives, as has been reported by other workers (Kaiser *et al.*, 1976; Young *et al.*, 1986; Nicoll-Griffith *et al.*, 1988; Baillie *et al.*, 1989). This was solved by the use of the Chiralpak AD CSP which gave baseline separation for both enantiomers of hydroxyibuprofen and all four stereoisomers of carboxyibuprofen.

Also, unlike alternative CSPs such as those based on proteins, polysaccharide columns have a relatively high loading capacity so that larger amounts of material could be injected on-column without appreciable loss of resolution. This, coupled with the better than baseline resolution of the four carboxyibuprofen stereoisomers, enabled on-column injections of up to 500 µg of the racemate. In this manner milligram quantities of the individual diastereoisomers could be isolated from the analytical column and this allowed the chiroptical properties to be recorded for the individual isomers for the first time. It was hoped that the stereochemical configuration could be assigned on the basis of the CD spectra alone. However, although differences in the CD bands were observed at the 225 nm band, which corresponded to the $n \rightarrow \pi^*$ transition of the carbonyl group, the stereochemical assignment could not be carried out with certainty. This was only solved by a combination of stereoselective synthesis (carried out by Dr. K. Afarinkia of the Chemistry Department, King's College London), chromatographic separation using a polysaccharide chiral CSP and isolation of the "*S* metabolites" after administration of (*S*)-ibuprofen. After assigning the stereochemistry, it was clear that the newly formed chiral centre in the isobutyl moiety had a Cotton effect in a similar direction at the 225 region but of smaller intensity compared to the original chiral centre, for a similar stereochemical configuration.

For the urine analysis of hydroxyibuprofen and carboxyibuprofen, the use of the sequential achiral-chiral chromatography approach ensured that only "clean" samples were introduced into the Chiralpak AD column, thus ensuring long term stability, efficiency and reproducibility. Although an extra chromatographic step was introduced into the methodology, sample clean-up steps were simplified, so that only a one-step solvent extraction was necessary. Also, time spent for the development of sample clean-up procedures were reduced. This procedure has the potential to be coupled and automated on-line. With the use of a switching valve, the eluent from the normal-phase column containing the peaks of interest can be diverted onto the CSP without detriment to chromatographic resolution, as the mobile phases of both systems are compatible, differing only in the ethanol content. The solvent strength of the achiral phase is much weaker (2 % v.v ethanol)

compared to that of the chiral phase (8% v/v ethanol). Hence diverting 1 - 2 ml of the achiral mobile phase onto the CSP should not affect resolution as there would be a concentration effect on the chiral column, resulting in peak sharpening.

8.3 Pharmacokinetic investigations of ibuprofen in the young

The development of enantioselective methods for the determination of ibuprofen and its metabolites in biological fluids facilitated pharmacokinetic studies following administration of the racemic drug in human volunteers. The pharmacokinetic data, based on serum, urinary and unbound drug concentrations enabled the stereoselectivity of various dispositional processes to be determined.

(a) Effect of plasma protein binding

The data presented in Chapter 6 derived from an examination of drug disposition in healthy young volunteers revealed enantioselective differences in the pharmacokinetics of ibuprofen, particularly in $t_{1/2}$, CL and V_d, as has been reported previously (Van Giessen and Kaiser, 1975; Lee *et al.*, 1985; Cox *et al.*, 1988; Avgerinos and Hutt, 1990). Before inherent stereoselectivity can be inferred, the effect of enantioselective protein binding has to be considered. The influence of plasma protein binding is obvious when the total and unbound CL and V_d are compared. As discussed in Chapter 6, the difference in V_d between the enantiomers was obviously due to protein binding, while there was evidence of stereoselectivity favouring the (*S*)- enantiomer in metabolic oxidation and glucuronidation Thus, because of the central modulating role of plasma protein binding, it is of utmost importance that unbound concentrations be determined in pharmacokinetic studies of the "profens".

A comparison of the magnitudes between CL and CL_u demonstrates that plasma protein binding is the limiting feature in the clearance of ibuprofen, as CL_u

is two orders of magnitude larger than CL. Therefore factors that decrease plasma protein binding would have a profound effect on drug clearance. However, because the inherent unbound clearance is unchanged, the increase in free drug concentration would only be transient, and at steady state, free drug concentration would remain unchanged despite increased total clearance (Aarons and Rowland, 1981). Of greater importance is if the unbound clearance is decreased as would be expected with hepatic disorders. In such a case, higher free concentrations would result, leading to enhanced pharmacological effects and possibly toxicity. It is also important to consider the ability of ibuprofen and other 2-APAs to displace other drugs from protein binding sites. *In-vivo* and *in-vitro* studies indicate that mutual displacement of the enantiomers of ibuprofen from protein binding sites occurs (Paliwal *et al.*, 1993; Evans *et al.*, 1989) resulting in an increased unbound fraction, with a corresponding increase in total CL and a lower AUC. Thus, the AUC of (*R*)-ibuprofen after a 400 mg oral dose of is significantly greater than the corresponding value after 800 mg of the racemic drug (Lee *et al.*, 1985), an observation also reported for flurbiprofen (Knadler *et al.*, 1989). Hence the enantioselectivity in the pharmacokinetics depends not only on the dose but also on the form administered i.e. individual enantiomer or racemate.

(b) Chiral inversion

The unidirectional chiral inversion process is unique to the 2-APA group of drugs and as such has been subject to much research attention as to its mechanism, extent and factors that affect it. The degree of inversion calculated in this study was 0.68 ± 0.07, and falls within the range of reported values of between 0.51 to 0.71. (Lee *et al*, 1985; Baillie *et al*, 1989; Geisslinger *et al*, 1990; Rudy *et al.*, 1991; Hall *et al*, 1993; Smith *et al.*, 1994). The wide range of values are probably a result of the different methods used to estimate F_{inv}. Also a wide variety of experimental designs were employed, leading to different estimates, which is a reflection of the complex processes involved in the enantiomeric disposition of ibuprofen. The fraction inverted estimated based on the AUCs of plasma-level time curves after

separate administration of the *R*- and *S*- enantiomers assumes that the clearance of the *S*- enantiomer is independent of (*R*)-ibuprofen, i.e. whether given alone or derived from the *R*- enantiomer via inversion. However, as mutually competitive protein binding displacement of enantiomers occurs *in-vivo* (Paliwal *et al.*, 1993), this assumption is not necessarily valid. The alternative method available when racemic doses are used is based on the urinary recovery method, as was used in the present study. Implicit in this method are the assumptions that all the dose is ultimately recovered in the urine and that the metabolites do not undergo inversion to an appreciable extent. Of an orally administered dose of radiolabelled ibuprofen, about 95% has been recovered in urine (unpublished observation by Boots Co. Ltd, Nottingham, U.K., cited by Lee *et al.*, 1985) and of this about 85% has been accounted for as ibuprofen and its major metabolites (Chapter 6). As such the first assumption of mass balance is not necessarily valid as the remaining *ca* 15% of the dose may have an influence on F_{inv}, albeit small. The second assumption is supported by *in-vitro* evidence that neither hydroxyibuprofen and carboxyibuprofen inhibit the formation of (*R*)-ibuprofenyl-CoA thioester (Tracy *et al.*, 1993; Hall *et al.*, 1993). However, this need not necessarily be true *in-vivo*. Theoretically, a method based on the administration of a pseudoracemic mixture would circumvent most of the problems encountered. However, a direct comparison between the pseudoracemic method and the urinary recovery method revealed that slightly higher but more consistent values were obtained with the urinary recovery method, the reasons behind this are not entirely clear (Rudy *et al.*, 1991). As such, these determined values of F_{inv} should be considered the best estimates of the magnitude of the inversion process under the experimental conditions involved. Of greater importance is the fact that the interindividual variation in the extent of inversion is relatively small, as shown in this study as well as others (Lee *et al*, 1985; Smith *et al*, 1994), indicating that the chiral inversion is a relatively "stable" process, i.e. the fraction of the dose presented to the body in the active form is relatively predictable in healthy subjects.

Although chiral inversion is generally accepted to be a unidirectional process, experiments in rats dosed with 2-phenylpropionic acid have shown

inversion in the reverse direction from *S*- to *R*- (Fournel and Caldwell, 1986). Recently, indirect evidence of this "reverse inversion" of ibuprofen in humans was provided in the form of the discovery of taurine conjugates in urine (Shirley *et al.*, 1994). A pseudoracemate containing (*S*)-[$^2H_3$]-ibuprofen was administered and taurine conjugates predominantly consisting of (*S*)-[$^2H_3$]-ibuprofen were found in urine. As taurine conjugates are formed via the formation of the CoA-thioester intermediate, this indicates that (*S*)-ibuprofen forms the CoA-thioester *in-vivo*, although this has not been reported *in-vitro* (Tracy *et al.*, 1993; Menzel *et al.*, 1994). In addition, small amounts of labelled (*R*)-ibuprofen and (*R*)-ibuprofen-taurine conjugate were found, providing evidence of *S* to *R* inversion. Thus, it appears that the "unidirectional" chiral inversion process is the net result of the competing rate processes involved. The "reverse" inversion appears to be relatively unimportant because of the much slower rate of thioester formation and of the other competing pathways stereoselective for (*S*)-ibuprofen, like taurine conjugation. Glycine conjugates of (*S*)-2-phenylpropionic acid have also been reported in dogs, where chiral inversion of the 2-phenylpropionic acid is known to occur to an equal extent in both directions (Tanaka *et al.*, 1992).

(c) Inversion and non-inversion clearance

From the findings in the present study, it is clear that chiral inversion is the main route of clearance of (*R*)-ibuprofen in man, as reflected by the relative magnitudes of $CL_{inv,\ u}$ which was two fold greater than $CL_{other,u}$. As $CL_{inv\ u}$ determines the amount of (*R*)-ibuprofen that has been converted to the active form, factors which affect this pathway are of clinical importance. *In-vitro* studies have provided evidence that the rate-limiting step for chiral inversion appears to be the formation of the (*R*)-ibuprofen thioester (Knights and Jones, 1992). More recent evidence has indicated that the formation of (*R*)- ibuprofen-adenylate, prior to thioesterification, may be the actual rate-limiting step. (Menzel *et al.*, 1994). This process is modulated by hepatic long chain fatty acid CoA synthetase (Muller *et al.*, 1992). Thus, factors which modulate the activity of these enzymes would affect

inversion. Increased inversion has been shown to occur in rats following administration of clofibrate (Knights *et al*, 1991), while palmitic acid (Muller *et al.*, 1992) pivalic acid (Xiaotao and Hall, 1993) and valproic acid (Tracy *et al.*, 1993) were inhibitors in the rat hepatocyte model. Theoretically, inhibition of the non-inversion clearance of (*R*)-ibuprofen would also increase chiral inversion. Proof of this is provided by *in-vitro* experiments using rat hepatocytes in which the broad spectrum cytochrome P_{450} inhibitors metapyrone and proadifen (SKF 525A) reduced the oxidation of (*R*)-ibuprofen and also increased fractional inversion (Xiatao and Hall, 1993). The effect of disease, especially hepatic dysfunction is another possible influence. To date decreased clearance of both enantiomers of ibuprofen has been reported in patients with liver cirrhosis although it is not clear whether the decrease in CL of (*R*)-ibuprofen is a result of a decrease in inversion and/or alternative pathways. Also plasma protein binding was not studied (Li *et al.*, 1993), which is important as liver cirrhosis is usually associated with a decline in plasma albumin levels (Dawling and Crome, 1989). Evidence from experiments using hepatocytes from experimentally (streptozocin) induced type I diabetic and genetically (type II) diabetic rats reveal that greater fractional inversion of ibuprofen and increased exposure to the CoA- thioesters occurs, implying that diabetic patients may be at greater risk of adverse effects associated with increase (*S*)-ibuprofen concentrations and hybrid triacylglycerol formation (Xiaotao and Hall, 1995). Thus, the effects of xenobiotics and disease on chiral inversion, either directly affecting the formation or hydrolysis of the CoA thioester or indirectly via modulation of the oxidative reactions of the *R*-enantiomer need to carefully studied in-view of the subsequent effects on unbound concentrations and the possibility of prolonged exposure to the CoA thioester.

8.4 Comparison of the enantioselective pharmacokinetics of ibuprofen between the young and elderly

Ageing is a natural phenomenon associated with changes occurring at the most basic and cellular level, leading to altered physiological responses and general

decline in the function of major organs. These changes will thus affect the ability of the body to handle administered drugs and cause changes in pharmacokinetics and pharmacodynamics. The physiological changes relevant to pharmacokinetics are summarised in Table 8.1 below:

Table: 8.1 Physiological changes relevant to pharmacokinetics

Process	Type of Interaction with age
Absorption	reduced gastric absorption
	reduced gastric emptying rate
	reduced GI motility
	reduced GI blood flow
Distribution	decreased body mass
	increased proportion of body fat
	decreased proportion of body water
	decreased plasma albumin
	decreased tissue perfusion
Metabolism	reduced liver mass
	reduced liver blood flow
	reduced hepatic metabolic capacity
Excretion	reduced glomerular filtration
	reduced renal tubular function
	reduced kidney blood flow
	reduced kidney mass

(Adapted from Dawling and Crome, (1989))

The way a particular drug will be affected by age will naturally depend on its properties with respect to protein binding, distribution, hepatic and renal clearance.

With the advent of stereospecific drug analysis, a further dimension has been added to the effect of ageing. In experimental animals, age related effects in enantioselective disposition of propranolol is well documented (Vermeulen *et al.*, 1992). In humans, stereoselective changes in the clearance of hexobarbital and mephobarbital can be expected with ageing (Chandler *et al.*, 1988; Hooper and Qing, 1990). With a drug such as ibuprofen, it would be expected that ageing would affect clearance, particularly with respect to clearance via oxidation with or without associated changes in stereoselectivity. Moreover, the extent of chiral inversion in the elderly has not been properly investigated. The pharmacokinetic parameters based on total (bound and unbound) drug concentrations did not show any significant differences between the two groups. Taken at face value, this would have easily led to an erroneous conclusion that there were no differences in the enantioselective disposition of ibuprofen between the young and elderly. However, because of the central role played by plasma protein binding in modulating the disposition of a highly bound drug like ibuprofen, the unbound concentrations need to be determined before any firm conclusions can be arrived at. Indeed, important and significant differences were observed with the protein binding, and hence AUC_u and CL_u for (*S*)-ibuprofen. Thus, the decrease in unbound clearance of the *S*- enantiomer was masked by the increased unbound fraction, resulting in no net change in total body clearance. Equally important is the fact that these changes are stereoselective, affecting the *S*- but not the *R*- enantiomer. Further evidence for these changes were provided by the reduction of unbound formation clearances of the metabolites derived from (*S*)-ibuprofen in the elderly, while formation clearances based on total drug concentrations did not show any change.

An increased unbound fraction together with a reduction in unbound clearance would mean that at steady state the total drug serum concentrations would remain the same but that the unbound concentration would increase. As it is the unbound concentrations that are important in determining pharmacological effect, the elderly are therefore potentially exposed to increased effect and risk of toxicity on a normal therapeutic dose. In extreme cases where unbound clearance is drastically decreased, a reduction in dose should be considered (Upton *et al.*, 1984).

Caution with dosing of ibuprofen in the elderly should also be exercised in view of the fact that the protein binding of the drug is dose dependent, with the unbound fraction increasing with increasing dose (Evans *et al.*, 1990; Smith *et al.*, 1994). The implications of this are that in elderly subjects, an increase in dose would lead to a disproportionate increase and accumulation of unbound (*S*)-ibuprofen concentrations, leading to increased risk of adverse effects such as GIT and haematological disturbances and renal toxicity. Evidence for this is provided from epidemiological studies which indicate that the elderly have a significantly higher odds-ratio of risk of gastrointestinal and other disorders with NSAIDs (Carson and Willet, 1993).

As ageing is associated with decreased liver perfusion and mass, hepatic metabolism would be expected to be reduced, especially for those drugs with a high hepatic extraction ratio, e.g. propranolol. However, ibuprofen is poorly extracted from the systemic circulation and coupled with the fact that the decrease in unbound clearance is stereoselective for the *S*- enantiomer, the underlying change seems to be due to lowered enzyme activity or affinity. There have been some suggestions that specific isoforms of the cytochrome P_{450} enzymes may be selectively impaired with ageing (Woodhouse and James, 1990). Results from *in-vitro* experiments show that ibuprofen is metabolised by the CYP 2C9 isoenzyme (Leemann *et al.*, 1993). However, to date no age-related effects on enzyme activity have yet been reported for this isoenzyme (Rudy *et al.*, 1995), and this would certainly have been the conclusion drawn form the present study had the unbound concentrations of the enantiomers not been determined.

The elderly are a very diverse population group. Ageing occurs at different rates, which may be affected by genetic predisposition e.g. type II diabetes, or by environmental factors and lifestyle. In addition the ageing process is often superimposed by various disease conditions to which the elderly are more susceptible e.g. renal dysfunction and musculoskeletal disorders. As a result of these multiple pathologies, the elderly are generally prescribed more drugs than the young. This co-medication would certainly give rise to interactions that affect drug

disposition, especially with interactions in protein binding. Thus the findings of this study should be considered with these factors in mind. Studies on patients with moderate renal dysfunction have demonstrated higher AUCs for (*S*)-ibuprofen and increased *S /R* ratios for serum concentrations, indicating that these patients have increased exposure to (*S*)-ibuprofen. This may exacerbate renal dysfunction by inducing ischaemia via prostaglandin inhibition (Chen and Chen, 1994). Unfortunately, the mechanism(s) underlying this condition were not investigated thoroughly. Moreover, these patients may be in greater risk than is obvious. In a separate study, elderly patients with mild renal dysfunction were found to have higher unbound concentrations of (*S*)-ibuprofen as compared to healthy elderly or young volunteers, even though the serum concentrations were "normal". This was found to be the result of decreased binding and concomitant reduction in unbound clearance in the elderly (Rudy *et al.*, 1995). Thus, elderly patients with moderate and severe renal dysfunction may face double risk of increased exposure to (*S*)-ibuprofen through higher serum as well as higher unbound concentrations. Also, in a study to identify predisposing factors for ibuprofen-related renal toxicity, advanced age and hypertension were identified as risk factors in patients with compromised renal haemodynamics (Chen and Chen, 1995).

The results of the present study provides evidence that the concurrent decrease in protein binding and unbound clearance of the active *S*- enantiomer occur in healthy elderly volunteers, and this result taken collectively with those of patients with underlying disease states indicate that the elderly are particularly at risk from the adverse effects of a seemingly relatively non-toxic drug like ibuprofen. It is also obvious that many more studies regarding the effect of disease states on the stereoselective disposition of ibuprofen and related 2-APAs in the elderly population are needed, in order to increase the safety and efficacy of these drugs in the ever increasing elderly population.

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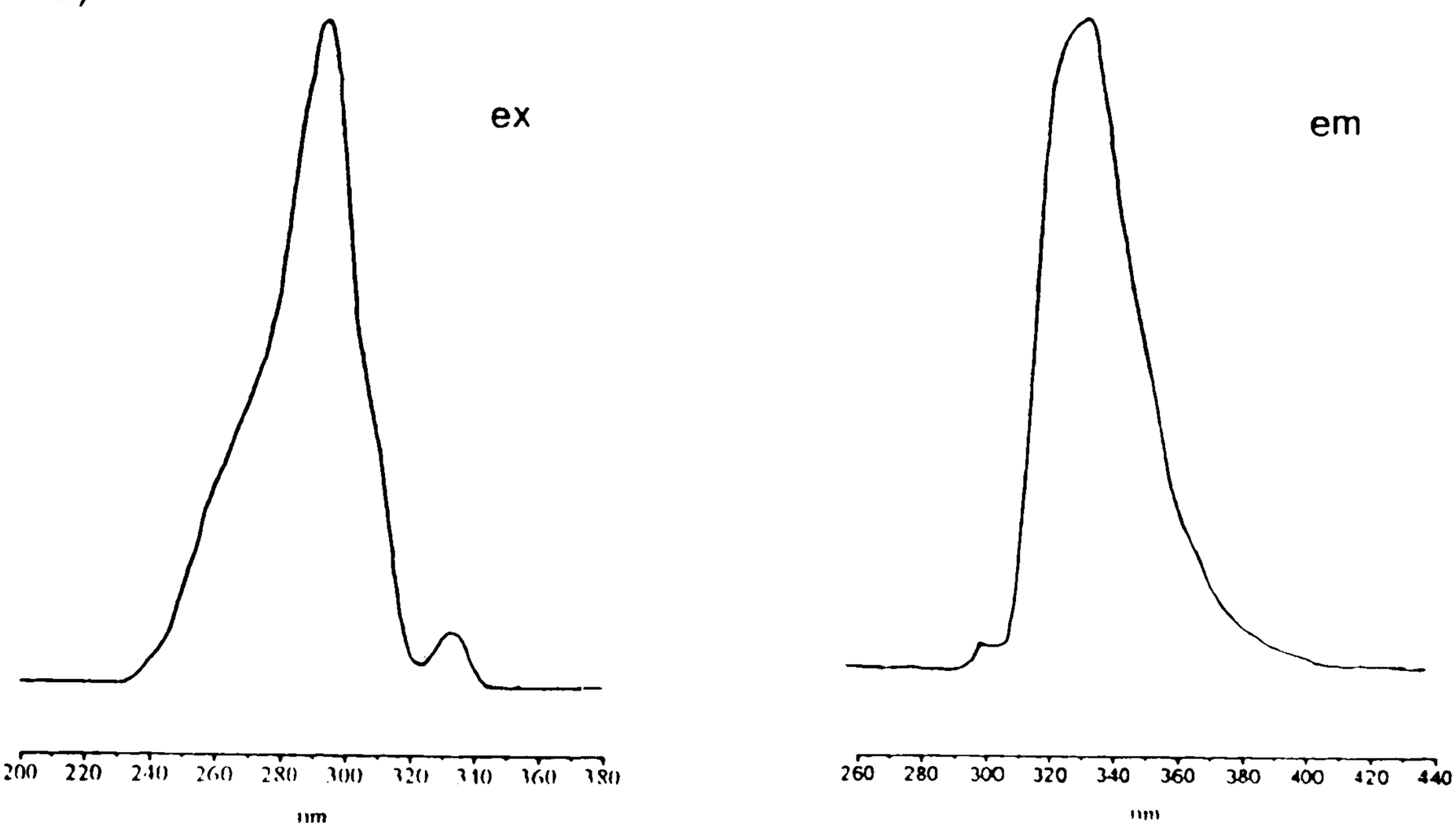
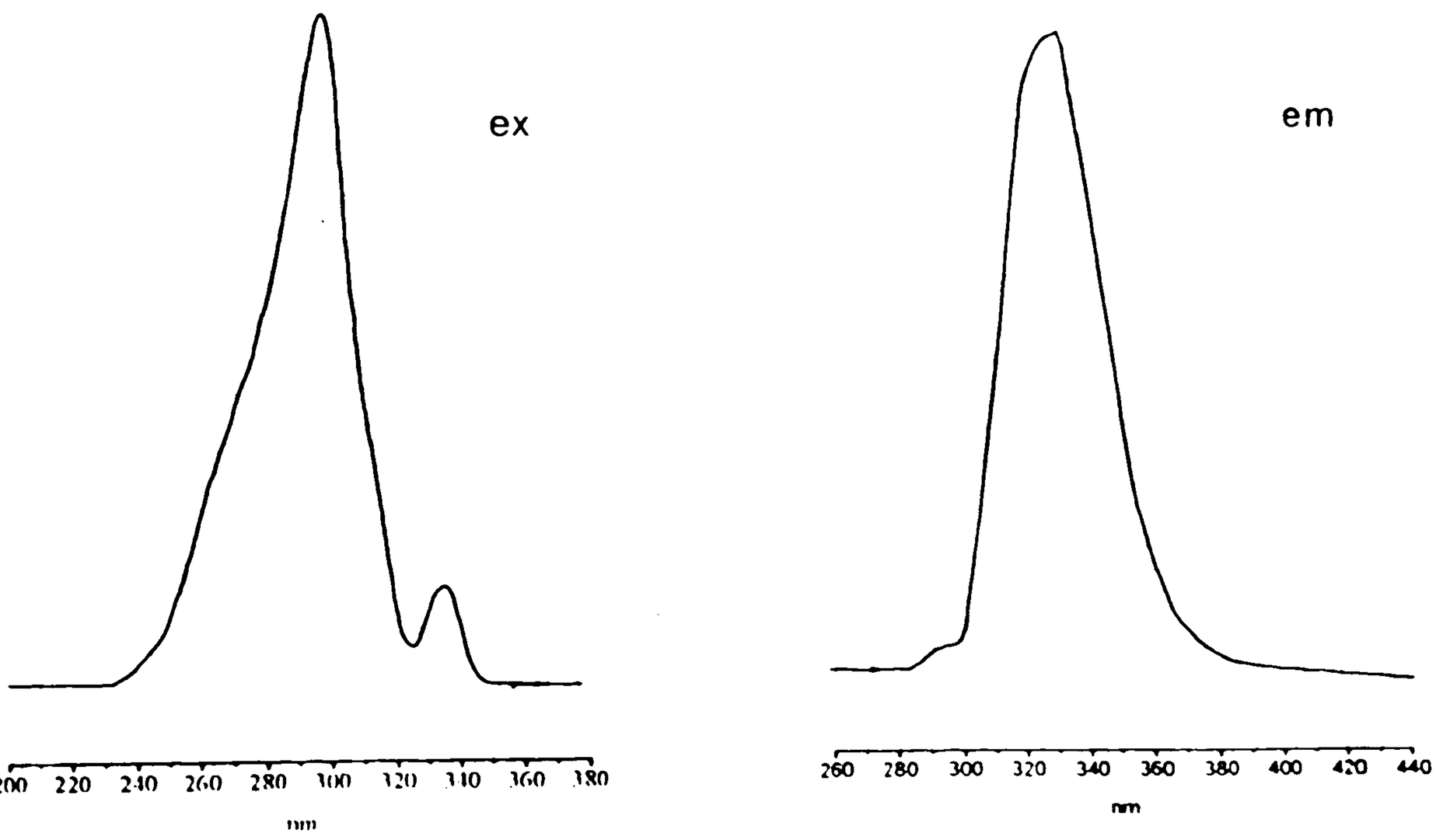
Appendix

Appendix 1: Fluorescence excitation (ex) and emission (em) spectra for (a) (*R*)-ibuprofenyl-(*R*)-1-(naphthen-1-yl)ethylamide (*R* acid:*R* amine) diastereoisomer and (b) (*S*)-ibuprofenyl-(*R*)-1-(naphthen-1-yl)ethylamide (*S* acid:*R* amine) diastereoisomer.

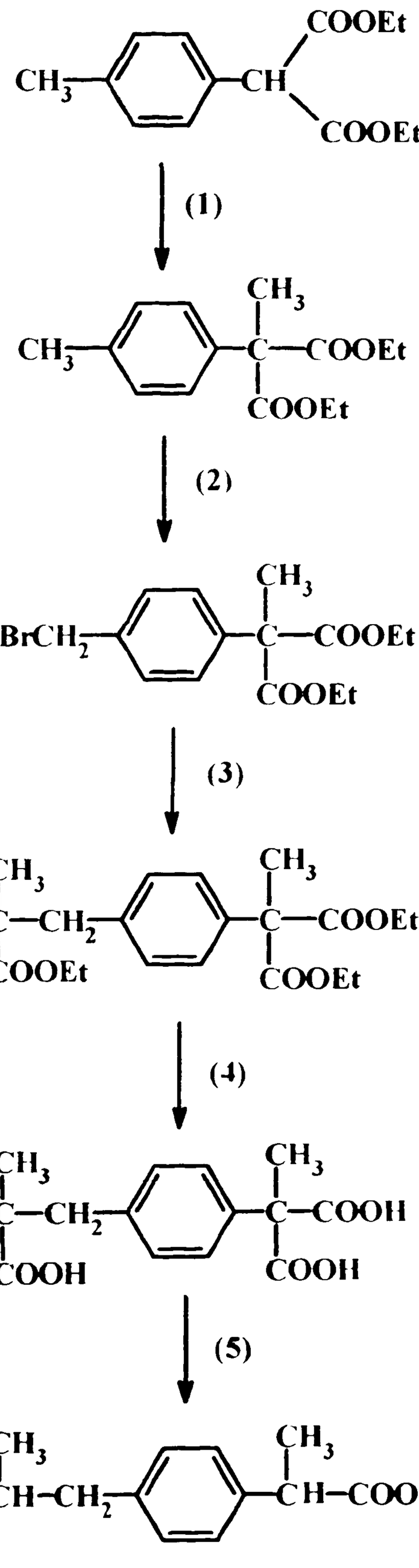
a)



b)



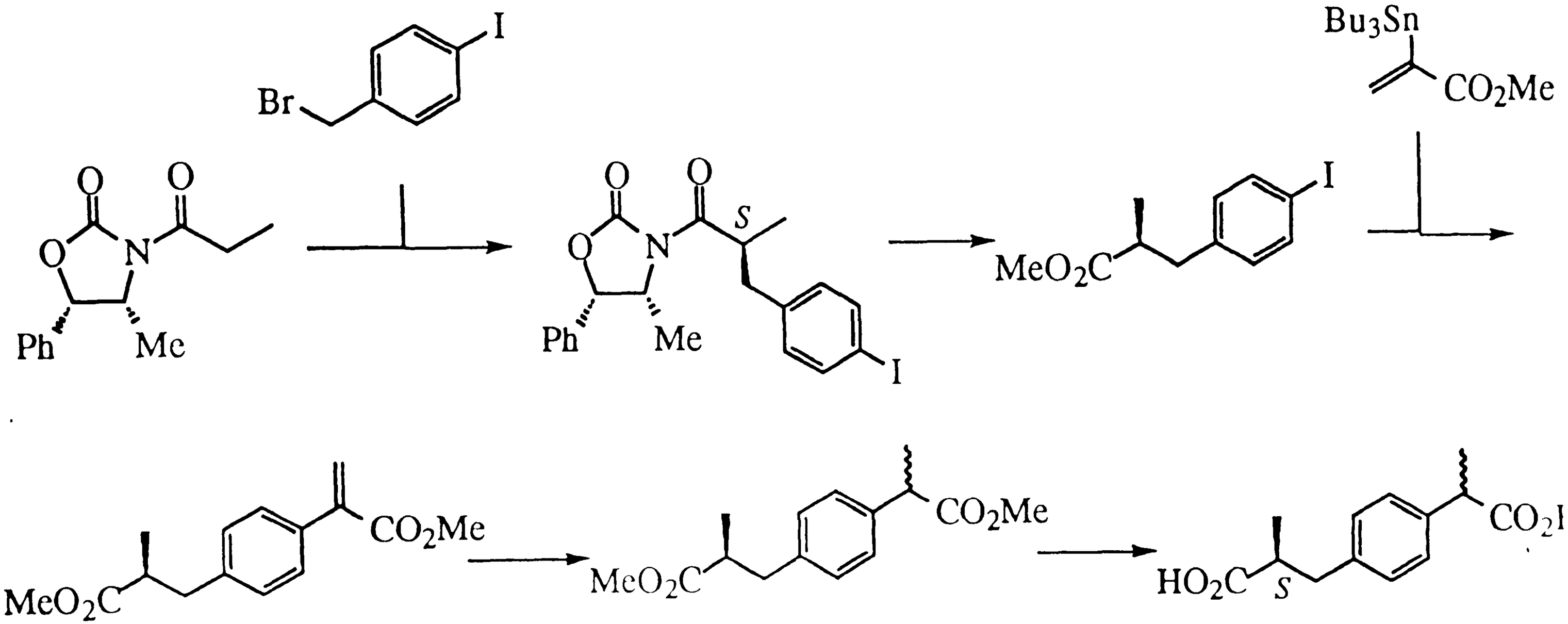
Appendix 2: Synthetic route adopted by Dr. J.A. Baker (Dept. of Pharmacy, University of Brighton) for the preparation of " racemic" (2'*RS*, 2*RS*)-carboxyibuprofen {(2'*RS*;2*RS*)-2-[4-(2-carboxypropyl)phenyl]propionic acid}. Reagents: (1) NaOEt, CH_3I; (2) N-bromosuccinimide, benzoylperoxide, CCl_4; (3) diethyl 2-methylmalonate, NaH, THF; (4) NaOH, EtOH 50%; H_2SO_4; (5) heat. Full details of the synthetic route will be published elsewhere.



Appendix 3: (a) Synthetic route adopted by Dr. K. Afarinkia (Department of Chemistry, King's College London) for the preparation of (2'*S*, 2*R*,*S*)-carboxyibuprofen diastereosiomers [(2'*S*, 2*R*,*S*)-2-[4-(2-carboxypropyl)phenyl]propionic acid).

Reagents: (1) LDA, THF, -78^{0}C; (2) butyllithium, anhydrous MeOH, 0^{0}C; (3) CuI, Pd (PPh_3), THF; (4) Pd/C, EtOH; (5) NaOH, 0^{0}C.

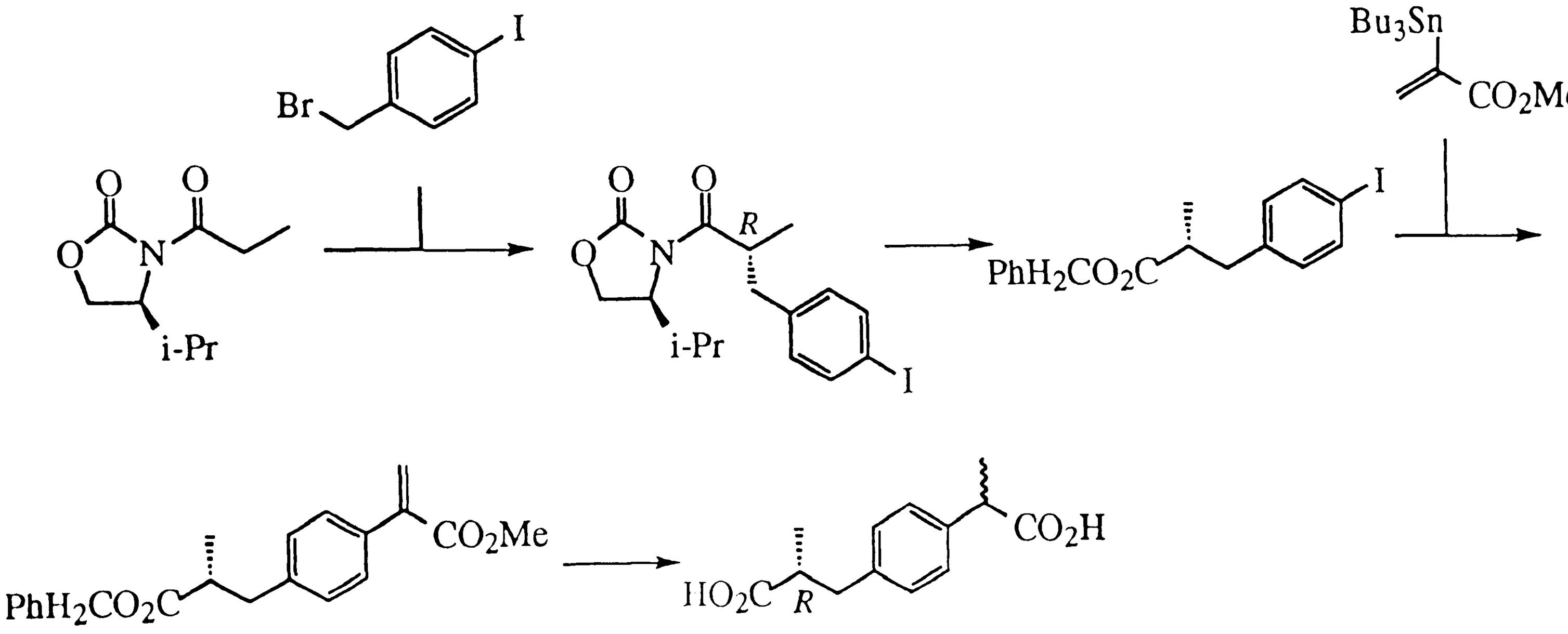
Full details of the synthetic route will be published elsewhere.



Appendix 3: (b) Synthetic route adopted by Dr. K. Afarinkia (Department of Chemistry, King's College London) for the preparation of (2'*R*, 2*R*,*S*)-carboxyibuprofen diastereosiomers [(2'*R*, 2*R*,*S*)-2-[4-(2-carboxypropyl)phenyl]propionic acid).

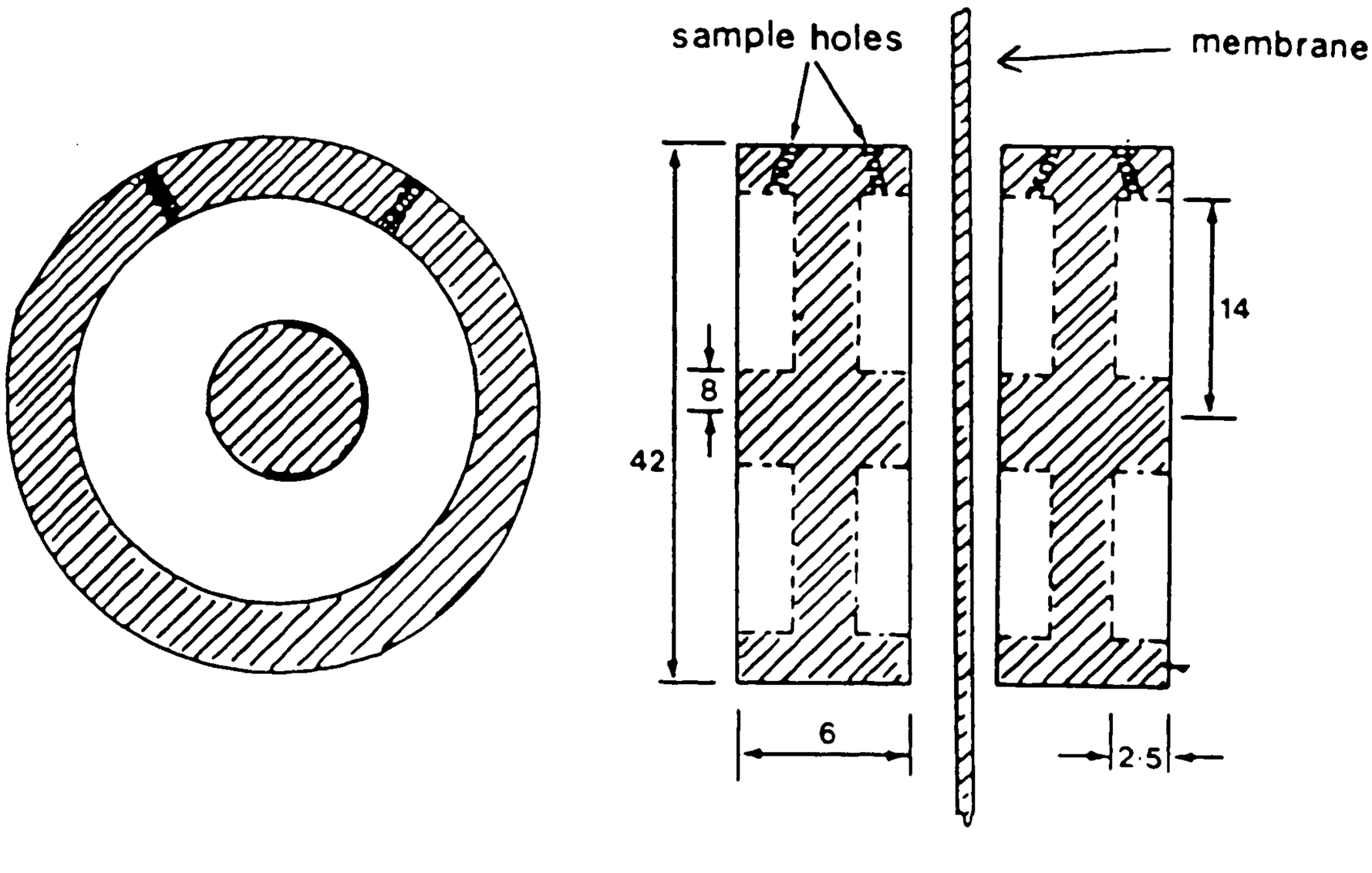
Reagents: (1) LDA, THF, -78^{0}C; (2) butyllithium, $PhCH_2OH$, THF, 0^{0}C; (3) CuI, Pd (PPh_3), dry DMF; (4) Pd/C, EtOH, EtOAC.

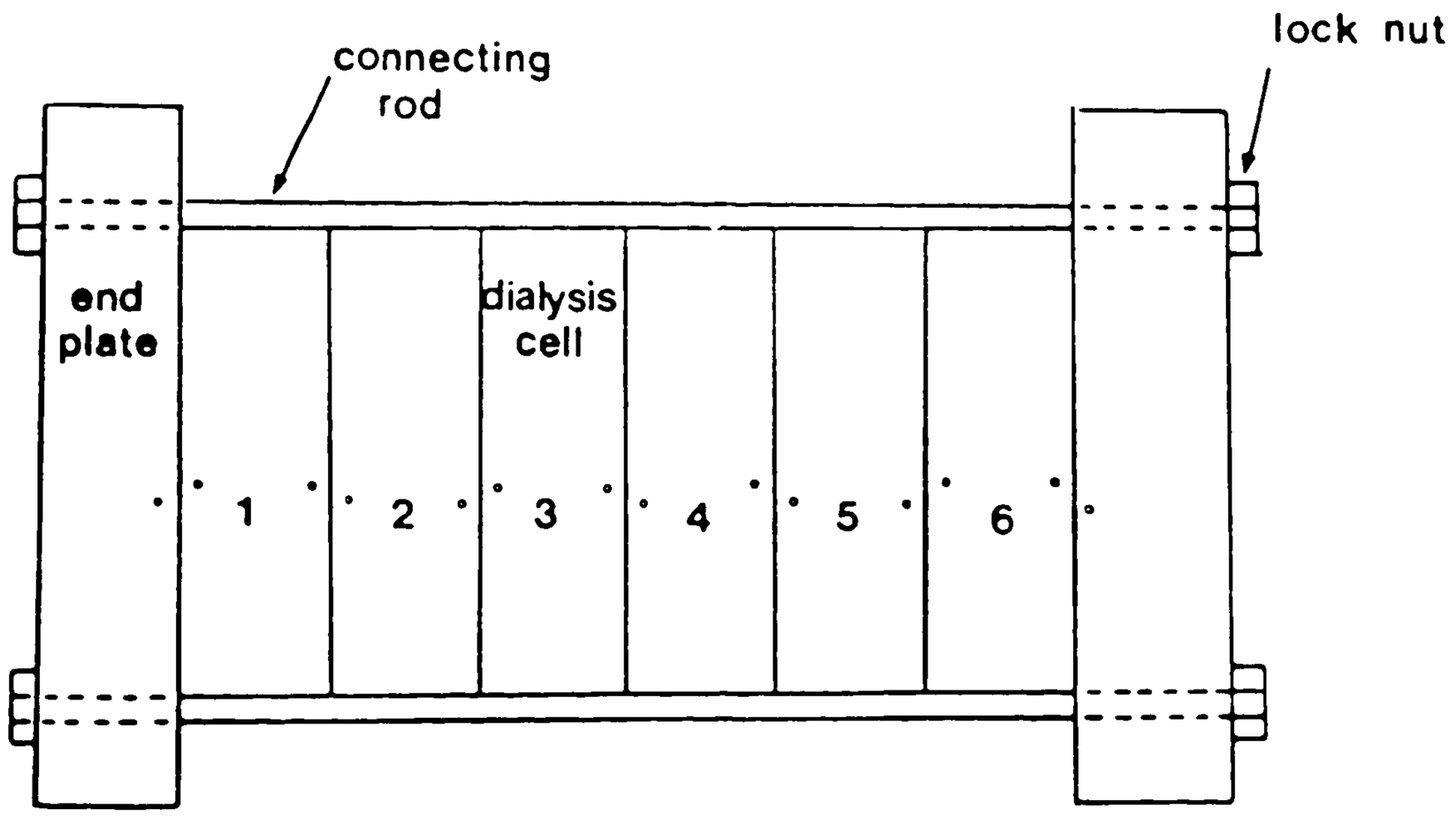
Full details of the synthetic route will be published elsewhere.



Appendix 4: Physical dimensions of perspex cells used in equilibrium dialysis.

The cells are made from 6 mm thick perspex sheet. (All dimensions in mm).





Appendix 5: Characteristics, serum albumin and creatinine concentrations for the young and elderly volunteers.

Young						
Subject	Sex	Age (yr)	Weight (kg)	Height (cm)	Albumin (g/L)	Creatinine (mg/dL)
s1	F	36	61	157	55.2	0.69
s2	M	36	59	175	58.4	0.84
s3	M	22	77	179	53.2	1.48
s4	F	29	68	163	53.2	0.93
s5	M	27	100	178	56.4	0.81
s6	F	28	68	163	60.4	0.65
s7	F	23	65	165	56.8	0.77
s8	M	24	62	169	53.6	0.68
mean	-	28.1	70.0	168.6	55.9	0.9
sd	-	5.4	13.4	8.0	2.6	0.3
Elderly						
es1	F	67	71	159	54.4	1.07
es2	F	67	80	168	54.4	0.88
es3	F	67	66	164	51.6	0.43
es4	M	78	76	169	51.6	1.18
es5	F	75	61	156	55.6	0.83
es6	M	68	81	171	56.4	1.09
es7	F	68	72	173	54	0.22
es8	M	66	69	169	53.2	0.71
mean	-	69.5	72.0	166.1	53.9	0.8
sd	-	4.4	6.8	6.0	1.7	0.3

Comparison of the means for serum creatinine, serum albumin, weight and height between the young and elderly was carried out using Student's t-test for independent samples, and the means are not significantly different ($p > 0.05$).

Appendix 6 : Serum concentrations (μg/ml) for (*R*)- and (*S*)-ibuprofen in individual healthy young volunteers.

	(*R*)-ibuprofen										
Time (hr)	s1	s2	s3	s4	s5	s6	s7	s8	Mean	S.D	CV %
0.25	0.00	0.15	8.26	0.21	2.86	0.96	0.36	8.35	2.64	3.61	136.60
0.50	0.49	0.26	8.28	2.16	7.15	10.47	6.47	18.41	6.71	6.03	89.90
0.75	0.36	2.45	8.32	3.02	8.77	18.45	8.34	19.20	8.61	7.04	81.68
1.00	3.22	4.69	11.61	6.14	10.75	21.38	10.21	20.76	11.09	6.85	61.70
1.50	6.94	13.03	17.64	14.61	13.22	20.38	10.40	20.30	14.57	4.72	32.39
2.00	17.70	11.90	19.52	18.83	13.29	18.25	12.19	16.28	15.99	3.10	19.37
2.50	16.57	10.17	16.54	21.64	13.56	16.70	10.24	11.96	14.67	3.93	26.80
3.00	11.42	8.56	13.52	20.93	8.45	11.51	8.41	8.77	11.45	4.28	37.36
3.50	10.74	7.83	11.27	14.26	6.23	8.60	8.04	6.84	9.23	2.68	29.04
4.00	9.13	6.56	9.82	12.02	4.64	7.80	7.83	7.43	8.15	2.21	27.15
6.00	2.76	3.41	1.65	3.00	1.42	1.66	2.53	1.50	2.24	0.77	34.57
8.00	0.94	2.50	0.74	1.70	1.14	0.54	0.81	0.45	1.10	0.69	62.30
10.00	0.14	1.13	0.00	0.83	0.09	0.25	0.69	0.00	0.39	0.43	110.29
24.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 6: (cont'd)

	(*S*)-ibuprofen										
Time (hr)	s1	s2	s3	s4	s5	s6	s7	s8	Mean	S.D	CV %
0.50	0.23	0.18	7.15	1.91	8.59	10.02	6.14	14.78	6.13	5.14	83.93
0.75	0.11	2.45	7.28	2.91	8.68	19.55	7.18	18.89	8.38	7.28	86.92
1.00	3.25	4.97	10.06	4.95	11.67	23.23	9.57	19.99	10.96	7.24	66.05
1.50	5.97	15.15	14.56	14.45	15.96	21.92	10.17	17.51	14.46	4.75	32.87
2.00	16.71	13.38	14.83	20.69	18.35	18.91	12.58	12.97	16.05	3.06	19.07
2.50	14.30	11.43	14.68	25.12	21.97	14.72	10.58	8.19	15.12	5.73	37.91
3.00	9.70	9.56	10.52	26.23	16.91	12.98	8.57	6.15	12.58	6.37	50.67
3.50	9.37	9.17	7.65	18.62	12.30	9.77	7.79	4.24	9.86	4.21	42.70
4.00	6.90	7.10	6.77	17.70	9.47	7.93	7.35	3.43	8.33	4.14	49.74
6.00	3.46	6.15	3.20	7.72	5.51	4.43	3.11	2.45	4.50	1.81	40.26
8.00	1.65	3.95	1.78	4.86	3.96	2.42	1.37	1.13	2.64	1.42	53.68
10.00	0.52	1.76	0.00	2.22	1.27	1.50	0.70	0.00	1.00	0.82	82.34
24.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 7: Cumulative urinary excretion of total ibuprofen enantiomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	*R*	0.28	0.50	0.61	0.66	0.68	0.70
	S	0.94	2.22	3.30	4.13	4.46	4.70
s2	*R*	0.09	0.44	0.63	0.71	0.75	1.05
	S	0.55	2.68	4.79	5.98	6.55	9.89
s3	*R*	1.01	1.39	1.67	1.82	1.82	1.82
	S	2.63	5.91	7.47	8.15	8.30	8.76
s4	*R*	0.29	0.62	0.75	0.83	0.85	0.89
	S	1.52	3.74	5.25	6.33	6.59	6.98
s5	*R*	0.21	0.92	1.15	1.35	1.44	1.44
	S	1.72	6.51	8.52	11.49	13.31	13.86
s6	*R*	0.26	0.61	0.87	1.27	1.42	1.42
	S	1.74	4.17	5.87	8.33	10.22	11.47
s7	*R*	0.60	0.87	0.90	0.95	1.00	1.01
	S	2.65	4.87	5.22	5.88	6.59	6.98
s8	*R*	0.49	0.49	0.68	0.73	0.80	0.85
	S	1.68	1.68	3.09	3.82	4.48	4.97
mean	*R*	0.43	0.63	0.87	1.00	1.05	1.15
	S	1.67	3.37	5.00	6.09	6.74	8.45
s.d.	*R*	0.29	0.41	0.36	0.41	0.41	0.38
	S	0.73	2.11	1.87	2.50	2.99	3.19

Appendix 8: Cumulative urinary excretion of ibuprofen enantiomers (as the glucuronide) in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	*R*	0.27	0.47	0.58	0.62	0.64	0.66
	S	0.88	2.02	3.10	3.91	4.24	4.47
s2	*R*	0.08	0.40	0.58	0.66	0.70	0.99
	S	0.52	2.47	4.48	5.64	6.20	9.48
s3	*R*	0.98	1.34	1.63	1.77	1.75	1.74
	S	2.52	5.70	7.21	7.86	7.98	8.27
s4	*R*	0.27	0.60	0.73	0.80	0.83	0.86
	S	1.40	3.53	5.01	6.08	6.34	6.71
s5	*R*	0.19	0.81	1.03	1.21	1.28	1.24
	S	1.60	5.92	7.80	10.71	12.50	12.95
s6	*R*	0.25	0.59	0.83	1.23	1.38	1.38
	S	1.70	4.04	5.69	8.11	9.98	11.22
s7	*R*	0.58	0.84	0.87	0.92	0.97	0.98
	S	2.58	4.76	5.09	5.73	6.43	6.80
s8	*R*	0.48	0.48	0.67	0.72	0.78	0.84
	S	1.57	1.57	2.87	3.60	4.25	4.70
mean	*R*	0.42	0.60	0.84	0.96	1.01	1.09
	S	1.60	3.22	4.78	5.85	6.49	8.07
s.d.	*R*	0.29	0.39	0.34	0.39	0.39	0.35
	S	0.71	2.00	1.75	2.35	2.84	3.01

Appendix 9: Cumulative urinary excretion of free ibuprofen enantiomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	*R*	0.01	0.03	0.04	0.04	0.04	0.04
	S	0.06	0.19	0.21	0.22	0.22	0.23
s2	*R*	0.01	0.04	0.05	0.05	0.05	0.06
	S	0.03	0.21	0.31	0.34	0.35	0.41
s3	*R*	0.03	0.05	0.05	0.05	0.05	0.09
	S	0.11	0.21	0.26	0.29	0.32	0.49
s4	*R*	0.02	0.02	0.02	0.03	0.03	0.03
	S	0.13	0.21	0.24	0.26	0.26	0.27
s5	*R*	0.02	0.11	0.13	0.14	0.16	0.21
	S	0.12	0.59	0.73	0.78	0.81	0.91
s6	*R*	0.01	0.02	0.03	0.04	0.04	0.04
	S	0.04	0.13	0.18	0.22	0.23	0.25
s7	*R*	0.02	0.03	0.03	0.03	0.03	0.03
	S	0.06	0.11	0.13	0.15	0.16	0.18
s8	*R*	0.01	0.01	0.01	0.01	0.01	0.01
	S	0.11	0.11	0.22	0.23	0.23	0.27
mean	*R*	0.01	0.03	0.03	0.03	0.04	0.06
	S	0.08	0.15	0.22	0.24	0.25	0.38
s.d.	*R*	0.01	0.03	0.03	0.04	0.05	0.06
	S	0.04	0.08	0.06	0.06	0.06	0.11

Appendix 10 : Cumulative urinary excretion of total hydroxyibuprofen enantiomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	*R*	0.25	0.47	1.51	3.02	4.12	5.28
	S	2.20	5.78	12.88	18.18	18.84	27.05
s2	*R*	0.07	0.56	1.70	2.69	3.36	4.23
	S	0.50	3.44	8.10	11.74	15.04	20.39
s3	*R*	0.30	0.68	1.33	2.14	2.03	2.66
	S	1.87	5.61	7.57	11.08	14.15	17.54
s4	*R*	0.18	0.46	1.47	3.34	3.05	3.02
	S	1.57	4.68	8.25	11.71	14.66	20.33
s5	*R*	0.15	0.89	1.77	2.08	3.46	5.46
	S	1.56	6.80	10.78	13.88	16.91	19.85
s6	*R*	0.66	1.19	2.13	2.39	2.23	2.70
	S	3.33	8.21	12.71	15.93	18.20	25.19
s7	*R*	0.68	0.88	1.49	1.98	2.28	2.64
	S	4.08	5.15	8.87	14.29	18.64	24.67
s8	*R*	0.36	0.36	0.77	1.54	1.61	2.57
	S	3.16	3.16	5.59	7.47	10.63	16.99
mean	*R*	0.33	0.73	1.52	2.40	2.77	3.57
	S	2.28	5.67	8.84	13.03	15.88	21.50
s.d.	*R*	0.23	0.27	0.42	0.59	0.86	1.24
	S	1.16	1.53	2.30	3.28	2.82	3.70

Appendix 11: Cumulative urinary excretion of hydroxyibuprofen enantiomers (as the glucuronide) in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	*R*	0.14	0.31	0.52	0.92	1.80	2.83
	S	1.87	5.26	10.84	13.30	13.43	21.42
s2	*R*	0.05	0.34	0.97	1.30	1.58	1.41
	S	0.45	2.77	5.99	7.70	9.84	13.58
s3	*R*	0.18	0.44	0.94	1.36	1.12	1.08
	S	1.66	5.10	6.62	9.42	11.97	14.13
s4	*R*	0.14	0.34	1.07	2.63	1.98	1.27
	S	1.44	4.32	7.61	10.32	12.96	16.64
s5	*R*	0.12	0.74	1.21	1.34	2.37	3.17
	S	1.49	6.41	9.53	12.25	14.58	15.69
s6	*R*	0.54	0.75	0.78	0.99	0.65	1.10
	S	3.02	7.08	8.99	10.60	12.14	17.33
s7	*R*	0.32	0.31	0.67	0.93	0.82	1.42
	S	3.16	3.67	6.64	10.26	13.03	18.65
s8	*R*	0.28	0.28	0.51	1.13	0.82	0.63
	S	2.94	2.94	4.82	6.29	8.29	11.40
mean	*R*	0.22	0.44	0.88	1.47	1.46	1.61
	S	1.81	4.27	7.17	8.97	11.03	16.10
s.d.	*R*	0.16	0.22	0.24	0.59	0.70	0.90
	S	1.13	2.23	1.66	2.47	3.00	3.14

Appendix 12: Cumulative urinary excretion of free hydroxyibuprofen enantiomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	*R*	0.11	0.16	0.99	2.10	2.32	2.45
	S	0.32	0.52	2.04	4.88	5.40	5.64
s2	*R*	0.02	0.22	0.73	1.39	1.77	2.81
	S	0.05	0.67	2.11	4.04	5.20	6.82
s3	*R*	0.12	0.24	0.39	0.78	0.91	1.58
	S	0.21	0.51	0.95	1.67	2.18	3.41
s4	*R*	0.04	0.12	0.40	0.72	1.06	1.75
	S	0.13	0.37	0.64	1.39	1.70	3.69
s5	*R*	0.03	0.15	0.56	0.75	1.09	2.29
	S	0.07	0.39	1.26	1.62	2.33	4.16
s6	*R*	0.12	0.44	1.35	1.40	1.58	1.59
	S	0.31	1.13	3.71	5.33	6.06	7.85
s7	*R*	0.36	0.57	0.81	1.06	1.46	1.22
	S	0.92	1.49	2.24	4.03	5.60	6.02
s8	*R*	0.08	0.08	0.25	0.41	0.79	1.95
	S	0.21	0.21	0.77	1.18	2.34	5.59
mean	*R*	0.11	0.27	0.69	1.07	1.37	1.96
	S	0.28	0.72	1.71	3.02	3.85	5.40
s.d.	*R*	0.11	0.17	0.36	0.54	0.51	0.53
	S	0.28	0.47	1.04	1.56	1.81	1.86

Appendix 13: Cumulative urinary excretion of total carboxyibuprofen stereoisomers in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Stereoisomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	2'*S*, 2*R*	0.32	1.23	3.54	5.25	4.70	4.99
	2'*R*, 2*R*	0.33	1.37	3.75	5.92	5.03	6.35
	2'*R*, 2*S*	1.10	4.29	10.59	15.29	17.55	17.96
	2'*S*, 2*S*	1.68	6.11	14.60	21.14	23.71	25.85
s2	2'*S*, 2*R*	0.12	1.01	2.07	2.78	2.88	2.58
	2'*R*, 2*R*	0.13	1.07	2.59	3.23	3.71	5.25
	2'*R*, 2*S*	0.40	3.60	8.37	12.21	15.21	16.51
	2'*S*, 2*S*	0.59	4.97	10.78	14.21	17.90	23.02
s3	2'*S*, 2*R*	0.19	0.80	1.40	2.02	2.15	2.61
	2'*R*, 2*R*	0.24	1.00	1.75	2.37	3.12	3.75
	2'*R*, 2*S*	0.67	3.14	5.53	8.26	10.00	12.97
	2'*S*, 2*S*	1.06	4.35	7.32	10.59	13.49	17.32
s4	2'*S*, 2*R*	0.10	0.48	1.26	2.09	2.24	2.92
	2'*R*, 2*R*	0.18	0.84	1.92	3.74	4.41	5.48
	2'*R*, 2*S*	0.37	2.09	5.39	8.86	10.38	12.94
	2'*S*, 2*S*	0.61	2.65	5.92	9.30	11.34	13.87
s5	2'*S*, 2*R*	0.18	1.24	2.09	2.70	3.03	3.58
	2'*R*, 2*R*	0.23	1.48	2.68	3.61	4.31	5.56
	2'*R*, 2*S*	0.66	4.55	7.91	10.28	13.00	12.68
	2'*S*, 2*S*	0.97	6.23	10.71	13.45	15.15	17.21
s6	2'*S*, 2*R*	0.47	1.18	1.64	1.83	3.08	1.80
	2'*R*, 2*R*	0.61	1.64	3.25	3.79	4.07	4.11
	2'*R*, 2*S*	1.67	5.33	9.74	12.89	14.59	21.55
	2'*S*, 2*S*	2.34	6.04	10.62	13.30	14.08	23.08
s7	2'*S*, 2*R*	0.62	0.74	1.29	2.16	4.14	1.96
	2'*R*, 2*R*	0.80	1.03	2.55	4.49	5.47	4.47
	2'*R*, 2*S*	2.20	3.36	7.63	15.26	19.61	23.45
	2'*S*, 2*S*	3.08	3.80	8.33	15.76	18.92	25.12
s8	2'*S*, 2*R*	0.40	0.40	0.89	1.66	1.63	1.85
	2'*R*, 2*R*	0.66	0.66	2.63	2.99	3.59	4.67
	2'*R*, 2*S*	1.68	1.68	6.20	9.80	12.89	13.52
	2'*S*, 2*S*	2.04	2.04	7.02	8.41	10.64	13.36
mean	2'*S*, 2*R*	0.30	0.95	1.77	2.56	2.98	2.79
	2'*R*, 2*R*	0.40	1.20	2.64	3.77	4.21	4.96
	2'*R*, 2*S*	1.09	3.76	7.67	11.61	14.15	16.45
	2'*S*, 2*S*	1.55	4.88	9.41	13.27	15.65	19.85
s.d.	2'*S*, 2*R*	0.19	0.26	0.42	0.41	0.76	0.62
	2'*R*, 2*R*	0.25	0.28	0.47	0.63	0.70	0.65
	2'*R*, 2*S*	0.68	1.03	1.50	2.31	3.03	4.17
	2'*S*, 2*S*	0.89	1.25	1.90	2.56	2.90	4.39

Appendix 14: Cumulative urinary excretion of carboxyibuprofen stereoisomers (as the glucuronide) in individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Stereoisomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	2'*S*, 2*R*	0.07	0.44	1.56	2.03	1.49	0.88
	2'*R*, 2*R*	0.15	0.75	2.14	2.94	2.33	2.88
	2'*R*, 2*S*	0.35	2.09	5.47	7.28	8.42	9.36
	2'*S*, 2*S*	0.59	3.13	7.58	9.91	11.12	9.89
s2	2'*S*, 2*R*	0.06	0.57	0.98	1.38	1.33	0.92
	2'*R*, 2*R*	0.09	0.62	1.33	1.48	1.71	2.68
	2'*R*, 2*S*	0.23	2.06	4.77	6.38	7.92	8.16
	2'*S*, 2*S*	0.34	2.83	6.40	6.71	8.79	11.50
s3	2'*S*, 2*R*	0.12	0.55	0.71	1.09	0.81	1.28
	2'*R*, 2*R*	0.18	0.78	1.13	1.44	1.98	2.35
	2'*R*, 2*S*	0.41	1.98	3.30	4.94	6.12	7.51
	2'*S*, 2*S*	0.72	3.09	4.42	6.40	7.71	9.38
s4	2'*S*, 2*R*	0.05	0.28	0.77	1.30	1.31	1.49
	2'*R*, 2*R*	0.13	0.55	1.18	2.52	2.84	3.40
	2'*R*, 2*S*	0.22	1.26	3.32	5.62	6.26	7.16
	2'*S*, 2*S*	0.33	1.77	3.88	6.23	7.39	7.41
s5	2'*S*, 2*R*	0.11	0.60	0.79	1.11	1.24	1.23
	2'*R*, 2*R*	0.16	0.80	1.30	1.65	1.87	2.80
	2'*R*, 2*S*	0.43	2.48	3.64	5.04	6.59	5.21
	2'*S*, 2*S*	0.62	3.29	5.12	6.24	6.81	7.14
s6	2'*S*, 2*R*	0.24	0.54	0.73	0.83	2.03	0.55
	2'*R*, 2*R*	0.38	0.84	1.66	1.83	2.00	1.39
	2'*R*, 2*S*	0.93	2.96	4.54	5.79	6.60	10.79
	2'*S*, 2*S*	1.30	3.12	5.25	6.22	5.93	11.88
s7	2'*S*, 2*R*	0.29	0.25	0.61	1.00	2.73	0.60
	2'*R*, 2*R*	0.47	0.43	1.36	2.20	2.70	1.24
	2'*R*, 2*S*	1.12	1.55	3.75	6.98	8.89	11.01
	2'*S*, 2*S*	1.57	1.57	4.32	7.48	8.00	12.32
s8	2'*S*, 2*R*	0.25	0.25	0.04	0.69	0.48	0.68
	2'*R*, 2*R*	0.35	0.35	1.29	0.98	1.04	1.39
	2'*R*, 2*S*	0.83	0.83	1.86	4.27	5.66	5.37
	2'*S*, 2*S*	1.24	1.24	3.34	3.03	4.15	5.29
mean	2'*S*, 2*R*	0.15	0.46	0.77	1.18	1.43	0.95
	2'*R*, 2*R*	0.24	0.68	1.42	1.88	2.06	2.27
	2'*R*, 2*S*	0.57	2.06	3.83	5.79	7.06	8.07
	2'*S*, 2*S*	0.84	2.69	5.04	6.53	7.49	9.35
s.d.	2'*S*, 2*R*	0.46	0.68	0.95	1.31	1.43	2.53
	2'*R*, 2*R*	0.14	0.15	0.16	0.48	0.56	0.78
	2'*R*, 2*S*	0.33	0.56	0.89	0.85	1.05	2.16
	2'*S*, 2*S*	0.46	0.68	0.95	1.31	1.43	2.53

Appendix 15: Cumulative urinary excretion of free carboxyibuprofen stereoisomers for individual healthy young volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Stereoisomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
s1	2'*S*, 2*R*	0.25	0.79	1.98	3.22	3.21	4.11
	2'*R*, 2*R*	0.19	0.62	1.61	2.98	2.71	3.47
	2'*R*, 2*S*	0.75	2.20	5.12	8.01	9.13	8.60
	2'*S*, 2*S*	1.09	2.98	7.02	11.23	12.58	15.96
s2	2'*S*, 2*R*	0.06	0.44	1.09	1.40	1.55	1.65
	2'*R*, 2*R*	0.05	0.44	1.26	1.76	2.00	2.57
	2'*R*, 2*S*	0.16	1.53	3.60	5.83	7.28	8.35
	2'*S*, 2*S*	0.25	2.15	4.37	7.50	9.10	11.52
s3	2'*S*, 2*R*	0.07	0.25	0.68	0.94	1.35	1.33
	2'*R*, 2*R*	0.07	0.22	0.62	0.93	1.14	1.40
	2'*R*, 2*S*	0.26	1.16	2.24	3.33	3.88	5.45
	2'*S*, 2*S*	0.33	1.26	2.90	4.19	5.78	7.95
s4	2'*S*, 2*R*	0.05	0.20	0.49	0.79	0.93	1.43
	2'*R*, 2*R*	0.05	0.29	0.74	1.22	1.57	2.09
	2'*R*, 2*S*	0.16	0.84	2.08	3.24	4.11	5.79
	2'*S*, 2*S*	0.29	0.89	2.04	3.07	3.95	6.46
s5	2'*S*, 2*R*	0.07	0.63	1.30	1.59	1.79	2.36
	2'*R*, 2*R*	0.07	0.68	1.38	1.96	2.44	2.76
	2'*R*, 2*S*	0.23	2.07	4.26	5.25	6.41	7.47
	2'*S*, 2*S*	0.36	2.93	5.59	7.21	8.33	10.07
s6	2'*S*, 2*R*	0.23	0.64	0.91	0.99	1.05	1.25
	2'*R*, 2*R*	0.23	0.79	1.59	1.96	2.07	2.72
	2'*R*, 2*S*	0.74	2.37	5.20	7.10	7.99	10.76
	2'*S*, 2*S*	1.04	2.92	5.37	7.09	8.14	11.21
s7	2'*S*, 2*R*	0.33	0.49	0.68	1.16	1.41	1.36
	2'*R*, 2*R*	0.33	0.61	1.19	2.29	2.77	3.23
	2'*R*, 2*S*	1.08	1.81	3.89	8.28	10.71	12.43
	2'*S*, 2*S*	1.52	2.23	4.01	8.27	10.92	12.79
s8	2'*S*, 2*R*	0.15	0.15	0.86	0.97	1.15	1.17
	2'*R*, 2*R*	0.30	0.30	1.34	2.01	2.55	3.28
	2'*R*, 2*S*	0.85	0.85	4.34	5.53	7.24	8.15
	2'*S*, 2*S*	0.80	0.80	3.68	5.38	6.49	8.07
mean	2'*S*, 2*R*	0.15	0.49	1.00	1.38	1.55	1.83
	2'*R*, 2*R*	0.16	0.52	1.22	1.89	2.16	2.69
	2'*R*, 2*S*	0.53	1.71	3.84	5.82	7.09	8.38
	2'*S*, 2*S*	0.71	2.19	4.37	6.74	8.16	10.50
s.d.	2'*S*, 2*R*	0.10	0.17	0.26	0.28	0.29	0.39
	2'*R*, 2*R*	0.12	0.21	0.33	0.45	0.53	0.61
	2'*R*, 2*S*	0.36	0.52	1.06	1.71	2.18	2.34
	2'*S*, 2*S*	0.45	0.77	1.19	1.80	2.15	2.14

Appendix 16: Serum concentrations (μg/ml) for (*R*)- and (*S*)-ibuprofen in individual healthy elderly volunteers.

	(*R*)-ibuprofen										
Time (hr)	es1	es2	es3	es4	es5	es6	es7	es8	Mean	S.D	CV %
0.25	13.10	0.00	2.16	0.00	0.30	0.19	0.31	0.00	2.01	4.54	226.19
0.50	20.40	0.30	14.00	0.00	5.09	9.15	9.86	5.79	8.07	6.88	85.20
0.75	20.66	1.66	22.14	11.07	9.68	10.61	16.64	12.88	13.17	6.59	50.07
1.00	22.17	9.69	23.09	17.75	19.97	10.57	15.70	20.18	17.39	5.05	29.05
1.50	14.14	20.76	19.35	16.13	10.42	9.49	16.83	17.86	15.62	4.03	25.81
2.00	10.06	19.96	15.36	16.93	15.83	8.81	13.23	14.70	14.36	3.62	25.23
2.50	7.81	16.40	13.74	16.00	13.62	8.60	9.15	11.67	12.12	3.34	27.57
3.00	5.77	15.50	11.08	10.30	11.07	8.32	6.56	11.64	10.03	3.12	31.06
3.50	5.40	12.72	9.33	7.49	10.92	7.96	5.17	7.64	8.33	2.59	31.06
4.00	4.60	11.64	7.40	6.42	7.85	6.33	5.08	7.48	7.10	2.16	30.49
6.00	1.48	4.91	2.88	5.98	3.83	1.43	1.56	4.51	3.32	1.75	52.76
8.00	0.54	1.65	1.30	1.76	1.64	0.61	0.48	1.77	1.22	0.58	47.37
10.00	0.15	0.70	0.23	0.27	1.06	0.42	0.42	0.92	0.52	0.34	64.36
24.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	282.84

Appendix 16 : (cont'd)

	(*S*)-ibuprofen										
Time (hr)	es1	es2	es3	es4	es5	es6	es7	es8	Mean	S.D	CV %
0.25	11.21	0.00	2.15	0.00	0.06	0.43	0.31	0.00	1.77	3.88	219.24
0.50	18.17	0.08	11.34	0.00	4.11	9.47	9.48	5.13	7.22	6.14	85.05
0.75	19.11	1.24	19.32	10.03	8.03	12.26	16.94	10.97	12.24	6.15	50.24
1.00	20.22	7.70	21.45	16.71	9.21	14.17	16.94	16.41	15.35	4.84	31.53
1.50	14.16	18.16	19.02	15.66	8.73	14.66	18.04	14.13	15.32	3.29	21.50
2.00	12.30	18.12	15.79	16.24	13.59	15.05	16.97	11.41	14.93	2.33	15.59
2.50	11.21	12.86	13.55	17.08	11.77	14.38	15.86	8.77	13.18	2.66	20.14
3.00	8.51	11.20	10.69	15.96	8.67	10.93	14.26	7.03	10.90	2.99	27.44
3.50	7.84	8.85	9.24	13.46	8.60	10.16	11.33	5.13	9.33	2.46	26.38
4.00	7.37	7.13	7.48	11.29	5.62	9.91	10.68	4.98	8.06	2.33	28.86
6.00	3.59	4.67	4.04	9.76	3.90	7.62	6.19	2.82	5.32	2.36	44.43
8.00	1.85	3.01	2.77	5.36	2.06	3.18	2.95	2.35	2.94	1.09	36.93
10.00	1.04	1.94	1.25	1.16	1.24	1.95	1.67	1.53	1.47	0.35	24.12
24.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.02	282.84

Appendix 17: Cumulative urinary excretion of total ibuprofen enantiomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	*R*	0.11	0.20	0.20	0.52	0.62	0.73
	S	1.26	2.49	2.49	7.43	9.15	11.09
es2	*R*	0.56	0.77	0.91	1.02	1.06	1.09
	S	5.21	7.41	8.77	9.74	10.39	12.58
es3	*R*	0.23	0.34	0.38	0.41	0.41	0.43
	S	2.58	4.12	4.67	5.43	6.11	7.12
es4	*R*	0.11	0.49	0.61	0.66	0.73	0.80
	S	0.97	2.17	3.06	3.77	4.49	5.66
es5	*R*	0.39	0.56	0.64	0.71	0.74	0.76
	S	3.90	5.77	6.72	7.58	8.25	8.50
es6	*R*	0.23	0.39	0.68	0.77	0.84	0.85
	S	1.72	3.03	5.57	6.50	7.29	8.14
es7	*R*	0.41	0.89	1.09	1.23	1.27	1.30
	S	2.05	4.51	6.06	7.30	7.89	8.46
es8	*R*	0.50	1.06	1.22	1.36	1.37	1.48
	S	3.06	4.58	5.23	6.19	6.23	8.77
mean	*R*	0.32	0.59	0.69	0.83	0.88	0.93
	S	2.59	4.26	5.01	6.74	7.48	8.79
s.d.	*R*	0.17	0.30	0.39	0.34	0.33	0.34
	S	1.43	1.75	2.61	1.75	1.87	2.17

Appendix 18: Cumulative urinary excretion of ibuprofen enantiomers (as the glucuronide) in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	*R*	0.11	0.18	0.18	0.47	0.57	0.68
	S	1.22	2.34	2.34	6.98	8.65	10.54
es2	*R*	0.52	0.73	0.86	0.96	1.01	1.04
	S	4.88	7.02	8.33	9.26	9.90	12.06
es3	*R*	0.22	0.33	0.36	0.39	0.40	0.41
	S	2.49	3.93	4.45	5.17	5.83	6.81
es4	*R*	0.11	0.47	0.58	0.64	0.70	0.77
	S	0.93	1.85	2.69	3.39	4.09	5.27
es5	*R*	0.37	0.53	0.61	0.68	0.70	0.73
	S	3.69	5.48	6.39	7.21	7.86	8.08
es6	*R*	0.20	0.35	0.64	0.73	0.79	0.80
	S	1.39	2.63	5.15	6.00	6.76	7.58
es7	*R*	0.38	0.84	1.03	1.16	1.20	1.23
	S	1.91	4.15	5.64	6.76	7.34	7.89
es8	*R*	0.47	1.02	1.18	1.32	1.33	1.44
	S	2.74	4.21	4.84	5.73	5.77	8.30
mean	*R*	0.30	0.56	0.66	0.79	0.84	0.89
	S	2.41	3.95	4.69	6.31	7.03	8.31
s.d.	*R*	0.16	0.29	0.38	0.33	0.32	0.33
	S	1.35	1.72	2.49	1.71	1.83	2.11

Appendix 19: Cumulative urinary excretion of free ibuprofen enantiomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	*R*	0.01	0.02	0.02	0.04	0.05	0.05
	S	0.04	0.15	0.15	0.45	0.50	0.56
es2	*R*	0.03	0.04	0.05	0.05	0.05	0.05
	S	0.33	0.39	0.45	0.47	0.49	0.52
es3	*R*	0.01	0.01	0.02	0.02	0.02	0.02
	S	0.09	0.19	0.22	0.26	0.28	0.31
es4	*R*	0.00	0.02	0.02	0.02	0.02	0.02
	S	0.04	0.32	0.36	0.38	0.40	0.40
es5	*R*	0.02	0.03	0.03	0.03	0.03	0.04
	S	0.21	0.29	0.33	0.37	0.39	0.42
es6	*R*	0.03	0.04	0.04	0.05	0.05	0.05
	S	0.33	0.40	0.42	0.50	0.53	0.56
es7	*R*	0.02	0.05	0.06	0.07	0.07	0.07
	S	0.14	0.36	0.42	0.54	0.55	0.57
es8	*R*	0.03	0.03	0.04	0.04	0.04	0.04
	S	0.33	0.37	0.39	0.46	0.46	0.48
mean	*R*	0.02	0.03	0.03	0.04	0.04	0.04
	S	0.19	0.31	0.32	0.44	0.46	0.48
s.d.	*R*	0.01	0.01	0.02	0.02	0.02	0.02
	S	0.13	0.09	0.15	0.09	0.09	0.09

Appendix 20: Cumulative urinary excretion of total hydroxyibuprofen enantiomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	*R*	0.21	0.51	0.51	3.36	4.54	6.02
	S	2.01	5.30	5.30	21.01	26.08	32.53
es2	*R*	0.59	1.08	1.31	1.56	1.90	4.75
	S	4.47	7.74	9.66	11.32	13.10	28.43
es3	*R*	0.19	0.41	0.50	0.93	1.48	2.16
	S	1.89	4.01	4.86	8.54	12.66	17.81
es4	*R*	0.18	0.52	0.84	1.33	1.71	4.54
	S	1.68	4.87	7.07	9.14	10.79	22.68
es5	*R*	0.33	0.63	0.79	1.14	1.39	2.44
	S	2.88	6.06	8.23	10.23	12.55	21.36
es6	*R*	0.33	0.68	1.68	2.21	2.86	4.02
	S	1.91	3.73	7.73	9.34	11.41	15.03
es7	*R*	0.33	1.06	1.62	3.60	4.59	7.35
	S	2.05	6.62	9.15	13.48	16.34	24.15
es8	*R*	1.13	1.82	2.43	3.88	4.04	5.17
	S	4.81	8.25	10.92	17.13	17.87	23.13
mean	*R*	0.41	0.84	1.31	2.25	2.81	4.56
	S	2.71	5.82	8.23	12.52	15.10	23.14
s.d.	*R*	0.32	0.47	0.66	1.20	1.39	1.73
	S	1.24	1.65	1.96	4.44	5.05	5.54

Appendix 21: Cumulative urinary excretion of hydroxyibuprofen enantiomers (as the glucuronide) in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	*R*	0.18	0.47	0.47	1.68	2.10	2.60
	S	1.87	5.06	5.06	16.26	19.58	23.70
es2	*R*	0.30	0.40	0.50	0.73	0.84	3.01
	S	3.68	5.98	7.53	9.05	10.33	24.03
es3	*R*	0.00	0.04	0.13	0.33	0.69	1.17
	S	1.13	2.64	3.47	6.19	9.55	13.58
es4	*R*	0.11	0.38	0.60	0.79	0.99	2.72
	S	1.37	4.24	6.15	7.67	9.01	18.97
es5	*R*	0.19	0.39	0.53	0.59	0.68	1.34
	S	2.40	5.20	7.30	8.36	10.12	17.32
es6	*R*	0.13	0.29	0.69	0.60	0.86	1.39
	S	1.28	2.65	5.42	5.83	7.15	9.53
es7	*R*	0.16	0.64	0.95	1.51	1.93	3.86
	S	1.48	5.11	7.17	9.02	10.84	17.26
es8	*R*	0.15	0.38	0.78	1.55	1.60	1.84
	S	2.07	4.40	6.48	10.83	11.28	13.87
mean	*R*	0.15	0.37	0.60	0.97	1.21	2.24
	S	1.91	4.41	6.22	9.15	10.98	17.28
s.d.	*R*	0.08	0.17	0.26	0.52	0.58	0.96
	S	0.83	1.21	1.42	3.30	3.70	5.00

Appendix 22: Cumulative urinary excretion of free hydroxyibuprofen enantiomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Enantiomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	*R*	0.03	0.05	0.05	1.68	2.44	3.42
	S	0.14	0.24	0.24	4.75	6.50	8.83
es2	*R*	0.30	0.68	0.81	0.83	1.05	1.74
	S	0.79	1.76	2.13	2.27	2.78	4.40
es3	*R*	0.19	0.37	0.37	0.60	0.79	0.99
	S	0.76	1.37	1.39	2.36	3.11	4.23
es4	*R*	0.07	0.13	0.24	0.54	0.72	1.82
	S	0.31	0.63	0.92	1.47	1.78	3.71
es5	*R*	0.14	0.25	0.26	0.56	0.71	1.10
	S	0.48	0.86	0.93	1.87	2.43	4.04
es6	*R*	0.20	0.39	0.99	1.61	2.00	2.63
	S	0.63	1.08	2.30	3.52	4.25	5.50
es7	*R*	0.18	0.42	0.68	2.09	2.67	3.49
	S	0.57	1.51	1.98	4.47	5.50	6.88
es8	*R*	0.98	1.45	1.65	2.33	2.44	3.33
	S	2.75	3.86	4.45	6.30	6.59	9.26
mean	*R*	0.26	0.47	0.71	1.28	1.60	2.31
	S	0.80	1.41	2.01	3.37	4.12	5.86
s.d.	*R*	0.30	0.44	0.50	0.73	0.86	1.04
	S	0.81	1.10	1.21	1.68	1.88	2.21

Appendix 23: Cumulative urinary excretion of total carboxyibuprofen stereoisomers in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Stereoisomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	2'*S*, 2*R*	0.09	0.54	0.54	2.29	2.97	3.34
	2'*R*, 2*R*	0.20	0.78	0.78	2.88	3.77	4.29
	2'*R*, 2*S*	0.57	2.58	2.58	10.22	13.24	15.11
	2'*S*, 2*S*	0.46	2.91	2.91	12.13	15.82	17.77
es2	2'*S*, 2*R*	0.45	0.92	1.20	1.36	1.73	3.10
	2'*R*, 2*R*	0.95	1.94	2.60	3.00	3.93	7.25
	2'*R*, 2*S*	2.30	4.74	6.41	7.10	9.28	15.27
	2'*S*, 2*S*	2.68	5.48	7.13	8.07	10.17	17.55
es3	2'*S*, 2*R*	0.20	0.46	0.56	0.97	1.27	1.53
	2'*R*, 2*R*	0.39	0.88	1.13	1.94	2.63	3.59
	2'*R*, 2*S*	1.29	3.21	4.32	8.22	11.78	16.96
	2'*S*, 2*S*	1.59	3.63	4.71	8.32	11.70	16.36
es4	2'*S*, 2*R*	0.13	0.63	1.12	1.63	2.08	4.49
	2'*R*, 2*R*	0.18	0.92	1.58	2.32	2.94	6.20
	2'*R*, 2*S*	0.52	2.60	4.78	6.80	8.42	17.07
	2'*S*, 2*S*	0.66	3.29	5.49	7.97	9.85	19.61
es5	2'*S*, 2*R*	0.27	0.57	0.69	0.97	1.27	2.59
	2'*R*, 2*R*	0.46	1.01	1.28	1.89	2.57	6.19
	2'*R*, 2*S*	1.27	3.09	4.02	6.28	8.83	21.49
	2'*S*, 2*S*	1.53	3.39	4.32	6.42	8.72	19.24
es6	2'*S*, 2*R*	0.30	0.79	2.03	2.64	2.99	3.92
	2'*R*, 2*R*	0.35	0.93	2.33	3.05	3.55	5.16
	2'*R*, 2*S*	1.08	2.94	7.49	9.67	10.99	14.70
	2'*S*, 2*S*	1.55	3.79	8.78	17.17	22.44	26.86
es7	2'*S*, 2*R*	0.21	1.44	2.20	2.76	3.21	4.07
	2'*R*, 2*R*	0.27	2.11	3.18	4.01	4.66	5.89
	2'*R*, 2*S*	0.95	5.98	8.82	11.28	12.99	16.39
	2'*S*, 2*S*	1.15	6.77	9.84	12.62	14.49	18.24
es8	2'*S*, 2*R*	0.73	1.46	2.04	3.24	3.39	3.94
	2'*R*, 2*R*	1.29	2.50	3.45	5.43	5.67	6.43
	2'*R*, 2*S*	3.86	7.72	10.75	17.08	17.83	20.20
	2'*S*, 2*S*	4.48	8.85	12.29	19.47	20.33	22.98
mean	2'*S*, 2*R*	0.30	0.85	1.41	1.98	2.36	3.37
	2'*R*, 2*R*	0.51	1.38	2.22	3.07	3.72	5.62
	2'*R*, 2*S*	1.48	4.11	6.66	9.58	11.67	17.15
	2'*S*, 2*S*	1.76	4.76	7.51	11.52	14.19	19.83
s.d.	2'*S*, 2*R*	0.22	0.73	0.74	3.00	3.91	4.24
	2'*R*, 2*R*	0.36	0.86	0.99	3.41	4.42	4.49
	2'*R*, 2*S*	1.11	2.16	3.26	4.53	4.68	5.58
	2'*S*, 2*S*	1.26	2.30	3.49	5.54	6.14	5.65

Appendix 24: Cumulative urinary excretion of carboxyibuprofen stereoisomers (as the glucuronide) in individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Stereoisomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	2'*S*, 2*R*	0.00	0.19	0.19	0.24	0.20	0.25
	2'*R*, 2*R*	0.09	0.40	0.40	0.40	0.58	0.78
	2'*R*, 2*S*	0.14	0.88	0.88	1.63	1.98	2.51
	2'*S*, 2*S*	0.00	0.96	0.96	1.20	1.81	2.27
es2	2'*S*, 2*R*	0.28	0.32	0.30	0.34	0.29	0.84
	2'*R*, 2*R*	0.76	1.08	1.27	1.51	1.82	3.64
	2'*R*, 2*S*	1.47	1.47	1.47	1.56	1.56	3.02
	2'*S*, 2*S*	1.97	2.91	3.35	3.88	4.49	8.51
es3	2'*S*, 2*R*	0.08	0.12	0.15	0.38	0.60	0.74
	2'*R*, 2*R*	0.23	0.34	0.45	0.91	1.40	2.14
	2'*R*, 2*S*	0.66	1.02	1.53	3.71	6.25	10.70
	2'*S*, 2*S*	0.91	1.54	2.12	4.30	6.80	10.51
es4	2'*S*, 2*R*	0.09	0.25	0.39	0.53	0.74	2.57
	2'*R*, 2*R*	0.13	0.50	0.76	1.08	1.40	3.89
	2'*R*, 2*S*	0.34	1.21	2.14	2.99	3.83	10.60
	2'*S*, 2*S*	0.22	1.42	2.23	3.38	4.35	12.27
es5	2'*S*, 2*R*	0.16	0.26	0.30	0.35	0.44	0.91
	2'*R*, 2*R*	0.27	0.52	0.66	0.80	1.03	2.83
	2'*R*, 2*S*	0.85	1.67	2.07	2.75	3.77	10.06
	2'*S*, 2*S*	1.11	2.07	2.56	3.36	4.34	9.54
es6	2'*S*, 2*R*	0.09	0.30	0.79	0.94	0.98	1.55
	2'*R*, 2*R*	0.16	0.47	1.13	1.37	1.54	2.76
	2'*R*, 2*S*	0.47	1.35	3.36	4.08	4.44	7.10
	2'*S*, 2*S*	0.76	1.86	4.12	10.82	15.04	18.35
es7	2'*S*, 2*R*	0.08	0.47	0.67	0.69	0.72	1.26
	2'*R*, 2*R*	0.11	0.84	1.21	1.25	1.32	2.14
	2'*R*, 2*S*	0.54	2.45	3.61	3.69	3.80	5.48
	2'*S*, 2*S*	0.47	2.57	3.87	4.27	4.46	6.44
es8	2'*S*, 2*R*	0.05	0.05	0.05	0.20	0.21	0.45
	2'*R*, 2*R*	0.51	0.81	1.11	1.78	1.86	2.23
	2'*R*, 2*S*	1.19	2.01	2.40	3.79	3.94	4.42
	2'*S*, 2*S*	1.63	2.73	3.75	6.14	6.41	7.12
mean	2'*S*, 2*R*	0.10	0.25	0.38	0.46	0.52	1.07
	2'*R*, 2*R*	0.28	0.62	0.94	1.14	1.37	2.55
	2'*R*, 2*S*	0.71	1.51	2.37	3.02	3.70	6.74
	2'*S*, 2*S*	0.88	2.01	3.14	4.67	5.96	9.38
s.d.	2'*S*, 2*R*	0.05	0.26	0.29	0.48	0.57	0.88
	2'*R*, 2*R*	0.16	0.23	0.31	0.41	0.42	0.87
	2'*R*, 2*S*	0.34	0.67	1.12	1.29	1.75	3.80
	2'*S*, 2*S*	0.51	0.65	1.08	3.05	4.21	5.13

Appendix 25: Cumulative urinary excretion of free carboxyibuprofen stereoisomers for individual healthy elderly volunteers following the oral administration of 400 mg racemic ibuprofen (results expressed as a percentage of dose).

Subject	Stereoisomer	Time					
		0 - 2 h	0 - 4 h	0 - 6 h	0 - 8 h	0 - 10 h	0 - 24 h
es1	2'*S*, 2*R*	0.12	0.35	0.35	2.05	2.77	3.09
	2'*R*, 2*R*	0.11	0.38	0.38	2.48	3.19	3.51
	2'*R*, 2*S*	0.43	1.71	1.71	8.58	11.26	12.60
	2'*S*, 2*S*	0.66	1.95	1.95	10.93	14.01	15.50
es2	2'*S*, 2*R*	0.17	0.60	0.91	1.02	1.44	2.27
	2'*R*, 2*R*	0.19	0.85	1.33	1.50	2.11	3.61
	2'*R*, 2*S*	0.82	3.26	4.94	5.54	7.72	12.26
	2'*S*, 2*S*	0.71	2.57	3.78	4.19	5.68	9.04
es3	2'*S*, 2*R*	0.12	0.34	0.41	0.58	0.67	0.79
	2'*R*, 2*R*	0.17	0.54	0.67	1.04	1.23	1.44
	2'*R*, 2*S*	0.63	2.19	2.79	4.51	5.53	6.27
	2'*S*, 2*S*	0.68	2.09	2.59	4.01	4.90	5.85
es4	2'*S*, 2*R*	0.05	0.37	0.73	1.10	1.34	1.93
	2'*R*, 2*R*	0.05	0.43	0.82	1.23	1.54	2.31
	2'*R*, 2*S*	0.17	1.38	2.64	3.81	4.59	6.46
	2'*S*, 2*S*	0.44	1.87	3.26	4.60	5.50	7.33
es5	2'*S*, 2*R*	0.11	0.30	0.38	0.62	0.83	1.68
	2'*R*, 2*R*	0.18	0.48	0.62	1.09	1.54	3.36
	2'*R*, 2*S*	0.42	1.42	1.95	3.53	5.06	11.43
	2'*S*, 2*S*	0.42	1.32	1.77	3.06	4.38	9.70
es6	2'*S*, 2*R*	0.21	0.49	1.24	1.70	2.01	2.37
	2'*R*, 2*R*	0.19	0.46	1.20	1.68	2.01	2.40
	2'*R*, 2*S*	0.61	1.59	4.13	5.59	6.55	7.59
	2'*S*, 2*S*	0.80	1.92	4.66	6.35	7.40	8.51
es7	2'*S*, 2*R*	0.14	0.97	1.54	2.07	2.50	2.81
	2'*R*, 2*R*	0.17	1.28	1.97	2.76	3.34	3.75
	2'*R*, 2*S*	0.41	3.53	5.20	7.59	9.19	10.91
	2'*S*, 2*S*	0.68	4.20	5.97	8.35	10.04	11.80
es8	2'*S*, 2*R*	0.68	1.41	1.99	3.05	3.18	3.49
	2'*R*, 2*R*	0.78	1.69	2.34	3.66	3.81	4.20
	2'*R*, 2*S*	2.67	5.71	8.36	13.29	13.89	15.78
	2'*S*, 2*S*	2.85	6.12	8.54	13.33	13.91	15.86
mean	2'*S*, 2*R*	0.20	0.60	1.03	1.52	1.84	2.30
	2'*R*, 2*R*	0.23	0.76	1.28	1.93	2.35	3.07
	2'*R*, 2*S*	0.77	2.60	4.29	6.56	7.98	10.41
	2'*S*, 2*S*	0.90	2.76	4.37	6.85	8.23	10.45
s.d.	2'*S*, 2*R*	0.22	0.55	0.65	2.61	3.43	3.74
	2'*R*, 2*R*	0.27	0.64	0.73	3.29	4.22	4.51
	2'*R*, 2*S*	0.82	1.63	2.48	3.79	4.02	4.30
	2'*S*, 2*S*	0.84	1.74	2.57	4.08	4.45	4.39